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FIXATION OF CARBON DIOXIDE BY PLANT ROOTS THROUGH PHOSPHOENOLPYRUVATE CARBOXYLASE *

by W. A. JACKSON and N. T. COLEMAN **

INTRODUCTION

The ability of various plant tissues to fix CO_2 by non-photosynthetic mechanisms has been recognized for some time. The best known example is the dark acidification process in leaves of succulents. Characteristic increases in organic acids during the dark and decreases during light have been observed by many investigators [28]. Bonner and Bonner [5] observed that acid formation during the dark bore a linear relationship to CO_2 concentration from 0 to 0.1 per cent with malate showing the largest increase. Other experiments using $C^{14}O_2$ have shown conclusively the incorporation of CO_2 into organic acids during this process [26 27 25]. Although non-succulents do not vary as drastically in their organic-acid composition as a result of light and dark treatments, there is good evidence with many species that CO_2 fixation into organic acids, principally malate, may occur in leaf tissue in the dark [22].

Fixation of CO_2 by intact root tissue also has been reported [18 12 10 11] and in each case the majority of $C^{14}O_2$ was found in malate. Kursanov [15] has shown that absorption of CO_2 and bicarbonate by roots is a metabolic process not linked directly to the entry of water. Furthermore, CO_2 absorbed by the roots was transported to the leaves. Vlasyuk *et al.*[29] indicate that C supplied as $NaHC^{14}O_3$ was taken up by roots only when the soil was at neutral or alkaline reaction. Similar findings were reported by Scharrer and Jung [21].

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This communication reports the occurrence in roots, as well as other plant tissue, of a carboxylating enzyme with equilibrium characteristics strongly favoring the fixation of CO_2. The enzyme, already reported to be present in spinach [2], succulents [20 30] and wheat germ [24] directly carboxylates phosphoenolpyruvate with the formation of oxaloacetate and inorganic phosphate. With malic dehydrogenase present in the system malate, rather than oxaloacetate, accumulates [1 30]. Due to the low CO_2 requirement and favorable equilibrium conditions, the presence of the enzyme in root tissues provides a mechanism for utilization of CO_2 from the soil-root environment.

MATERIALS

Snap beans (*Phaseolus vulgaris*, var. Tendergreen) were germinated in quartz sand and grown for 12 to 14 days in the greenhouse, at which time roots were selected for preparation of extracts.

Bicarbonate solutions labelled with C^{14} were prepared by acidifying $BaC^{14}O_3$ obtained from Oak Ridge National Laboratories and trapping the evolved CO_2 in NaOH.

The tricyclohexylamine salt of phosphoenolpyruvic acid (PEP) was obtained from the California Foundation for Biochemical Research, while reduced diphosphopyridine nucleotide (DPNH) was generously donated by Dr. H. J. Evans. The barium salt of 3-phosphoglyceric acid (3PGA) was dissolved in HCl and, after removal of Ba as the sulfate, the acid was titrated to pH 7.4 with solid tris-(hydroxymethyl)-amino methane (Tris). Tris-acetate and Tris-pyruvate were prepared by directly titrating the free acids with Tris.

METHODS AND RESULTS

The reaction sequence may be illustrated as follows:

[a] PEP + HCO_3 → oxaloacetate + inorganic phosphorus

[b] Oxaloacetate + DPNH → malate + DPN.

Assay of activity by disappearance of DPNH

As the equilibrium for both reactions lies far to the right [23 6], an estimation of PEP carboxylase activity when both enzymes are present may be obtained by measuring spectrophotometrically the disappearance of DPNH at 340 mμ [24]. Enzyme extracts were prepared by grinding in a cold mortar 10g of 12-day old snap-bean roots with 40 ml of 0.05 *M* Tris buffer at pH 7.4. The macerated tissue was transferred to a Ten Brock homogenizer, ground for three

minutes and centrifuged at 20,000 × *g* for fifteen minutes at 0–2° C. The supernatant, used in these assays, was dialyzed against 0.01 *M* Tris buffer at pH 7.0 for four hours. The standard assay mixture contained 10 μmoles $MgCl_2$, 0.75 μmoles PEP and 0.09 μmoles DPNH. The total volume of 3 ml was adjusted by addition of 0.05 *M* Tris buffered at the desired pH. Reaction was started by the addition of 0.10 ml of dialyzed root extract. Enzyme activities are expressed as units per g fresh weight considering one unit to be a change of −0.001 in optical density measured from 30 seconds to 3 minutes and 30 seconds.

Oxidation of DPNH was increased by the addition of PEP or $KHCO_3$ either singly or in combination (Table 1). A comparison of

TABLE 1

Influence of PEP and $KHCO_3$ upon oxidation of DPNH by bean-root extracts. Reaction mixture containing 0.09 μmoles DPNH and 10 μmoles $MgCl_2$ per ml adjusted to 3 ml by .05 *M* Tris buffer, pH 7.0. Activity expressed in units (–0.001 OD) per g fresh weight

Treatment	Activity
Endogenous	152
PEP, 0.75 μmoles per ml	177
$KHCO_3$, 10 μmoles per ml	216
PEP (0.75 μmoles) + $KHCO_3$ (10 μmoles)	263

the K, Na and Tris salts of bicarbonate illustrated a slightly larger stimulation of DPNH oxidation by $KHCO_3$ than by $NaHCO_3$, although bicarbonate rather than the monovalent ions appeared to be the major requirement, since the Tris salt was equally as effective as $KHCO_3$ (Table 2).

TABLE 2

Influence of bicarbonate salts upon oxidation of DPNH by bean-root extracts. Reaction mixture contained 10 μmoles $MgCl_2$, 0.75 μmoles PEP and 0.09 μmoles DPNH per ml. Total volume adjusted to 3 ml by .05 *M* Tris buffer pH 7.0. Bicarbonate salts adjusted to pH 7 by bubbling CO_2 through the solutions. Activity expressed in units (–0.001 OD) per g fresh weight

Treatment	Activity	
	Exp. 1	Exp. 2
None	165	167
$KHCO_3$*	222	235
$NaHCO_3$*	217	207
Tris HCO_3*	236	—

* added at 10 μmoles per ml

Assay of activity by fixation of $C^{14}O_2$

Although indicating the existence of PEP carboxylase in the root tissue, the oxidation of DPNH is not entirely satisfactory for assays with non-purified enzyme preparations due to the large endogenous activity and the indirect nature of the measurement. As it is difficult to separate the influence of added components on the reaction rate from possible influences on the endogenous activities of the crude preparations, the activity of PEP carboxylase was more unambiguously evaluated by measuring fixation of $C^{14}O_2$.

The procedure was essentially a modification of that employed by Bandurski and Griener [2] and Saltman *et al.*[20]. Each ml of the standard reaction mixture contained 16.0 μmoles $MgSO_4$, 2.38 μmoles PEP, 16.0 μmoles Tris buffer pH 7.4, 4.96 μmoles $NaHC^{14}O_3$ and 0.16 ml root extract. Unless otherwise noted, the crude enzyme extract was prepared by grinding 1.5 g of fresh root tissue, previously equilibrated for 15 minutes with 1 per cent NH_4OH, with 1.0 volume of 0.1*M* Tris buffer pH 7.4 in a cold mortar. Coarse cell debris was separated by straining through cheesecloth and discarded. The assay was conducted in a water bath at 37°C, reaction being started by addition of the extract. At the end of the desired time, reaction was stopped and unreduced CO_2 liberated by addition of 0.1 ml of 1 *N* HCl. Aliquots of 0.1 ml were plated and counted with a windowless flow counter. Two identical reaction tubes were prepared for each treatment and duplicate aliquots were taken for counting. The amounts of $C^{14}O_2$ fixed were calculated from the difference between the original activity and that remaining after acidification and are expressed as μmoles CO_2 fixed per g fresh weight of tissue per unit time.

The procedure assumes that acidification and drying of the aliquot results in complete liberation of the unreduced carbon. To test the validity of this assumption, tubes were prepared without root extract and subjected to the standard assay procedure. Aliquots after acidification contained no activity in excess of background. The failure to find activity in the absence of PEP (Figure 2) further substantiates this assumption. As the procedure does not consider the dilution caused by CO_2 produced in endogenous respiratory activity or its subsequent refixation, the amount of $C^{14}O_2$ fixed is a minimum value for total fixation.

Figure 1 shows the rate of CO_2 incorporation during the assay

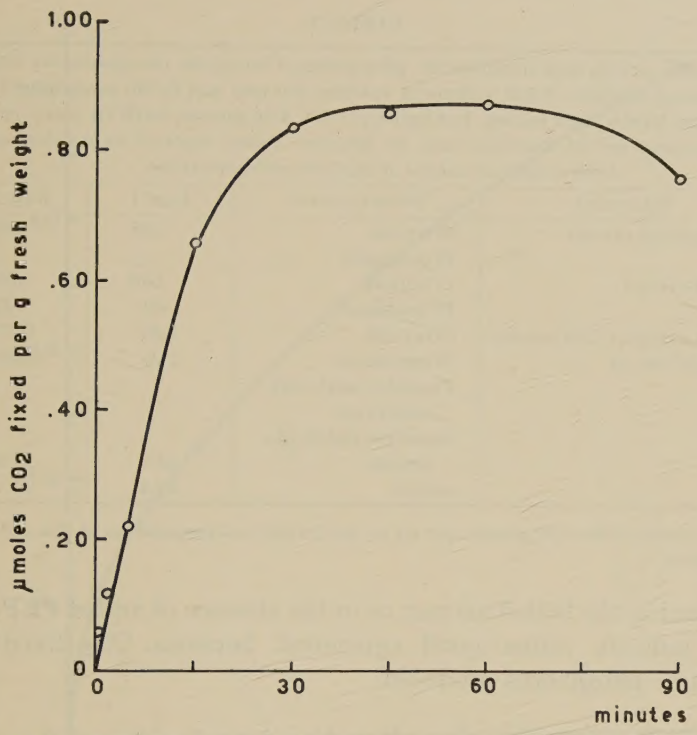

Fig. 1. Rate of CO_2 fixation by snap-bean root extracts. Total volume of standard reaction mixture was 0.63 ml and contained 16.0 μmoles $MgSO_4$, 2.38 μmoles PEP, 16.0 μmoles Tris buffer pH 7.4, 4.96 μmoles $NaHC^{14}O_3$ and 0.16 ml root extract per ml.

procedure. The reaction was essentially linear through 15 minutes and slowed considerably thereafter.

For every mole of PEP carboxylated, one mole of inorganic phosphorus should be produced (reaction *a*). Consequently, a measure of inorganic phosphorus appearance after a given time is also a measure of reaction rate. In order to compare inorganic phosphorus production with CO_2 fixation, two experiments were conducted using somewhat larger volumes of the assay mixture. Inorganic phosphorus was measured by the method of Ernster *et al.*[8]. Endogenous phosphatase activity was essentially nil in these preparations (Table 3) as no appreciable inorganic phosphorus was produced in the absence of added PEP. Moreover, exchange of $C^{14}O_2$ for non-labelled CO_2 was negligible also as very little fixation

TABLE 3

Fixation of CO_2 and simultaneous production of inorganic phosphorus by crude bean-root extracts. Total volume of reaction mixture was 2.3 ml containing 16.0 μmoles $MgSO_4$, 16.0 μmoles Tris buffer pH 7.4, 4.96 μmoles $NaHCO_3$ and 0.16 ml root extract per ml. Reaction time, 90 minutes. Values reported as μmoles per g fresh weight are means of duplicate determinations

Treatment	Value reported	Exp. I	Exp. II
Boiled root extract	CO_2 fixed	.005	.007
	Pi produced	—	—
Root extract	CO_2 fixed	.010	.009
	Pi produced*	.01	.00
Root extract + 2.38 μmoles PEP per ml	CO_2 fixed	1.56	0.77
	Pi produced*	1.39	0.56
	Percent of total radioactivity in:		
	aspartate and/or glutamate	48.7	47.9
	malate	23.4	11.1

* in excess of the 0.22 μmoles per ml in the boiled root preparation at the end of the experiment

occurred in the boiled extract or in the absence of added PEP. The data indicate rather good agreement between CO_2 fixed and inorganic phosphorus produced.

Substrate requirements

Figures 2 and 3 illustrate the dependency of CO_2 incorporation upon PEP and HCO_3 concentrations. Relatively low amounts of HCO_3 are sufficient to produce appreciable reaction rates.

Experiments conducted to determine if substrates other than PEP could be utilized by the bean-root homogenates in the CO_2 fixing reaction are reported in Table 4. No fixation occurred when pyruvate or acetate were substituted for PEP, indicating that PEP is not converted to these compounds prior to fixation. Further experiments showing CO_2 fixation when 3-PGA was added in the absence of fluoride, also indicate that PEP is carboxylated directly and not converted to acetate or pyruvate prior to carboxylation. Table 5 shows fixation at about one-fourth the PEP rate when 3-PGA was used as substrate, indicating the presence of phosphoglyceric mutase and enolase in the crude bean-root extracts. When fluoride, an inhibitor of the enolase system [14], was added, CO_2 fixation in the presence of 3-PGA was largely prevented, while little reduction of activity occurred with PEP as the substrate.

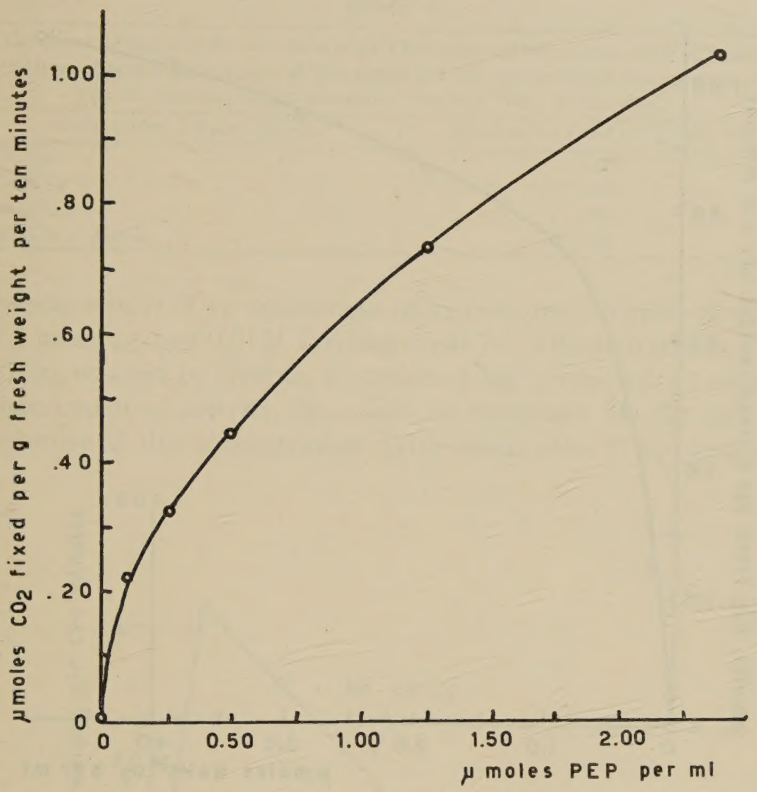

Fig. 2. Effect of PEP concentration upon CO_2 fixation by snap-bean root extracts. With the exception of the PEP variables, the standard reaction mixture (Figure 1) was used. Reaction time, 10 minutes.

TABLE 4

Comparison of acetate, pyruvate and phosphoenolpyruvate as substrates for the fixation of CO_2 by bean-root extracts. Standard reaction mixture. Reaction time, 10 minutes

Treatment	μmoles CO_2 fixed per g fresh weight
No substrate	.004
PEP*	.617
Acetate*	.005
Pyruvate*	.004

* added at 2.38 μmoles per ml

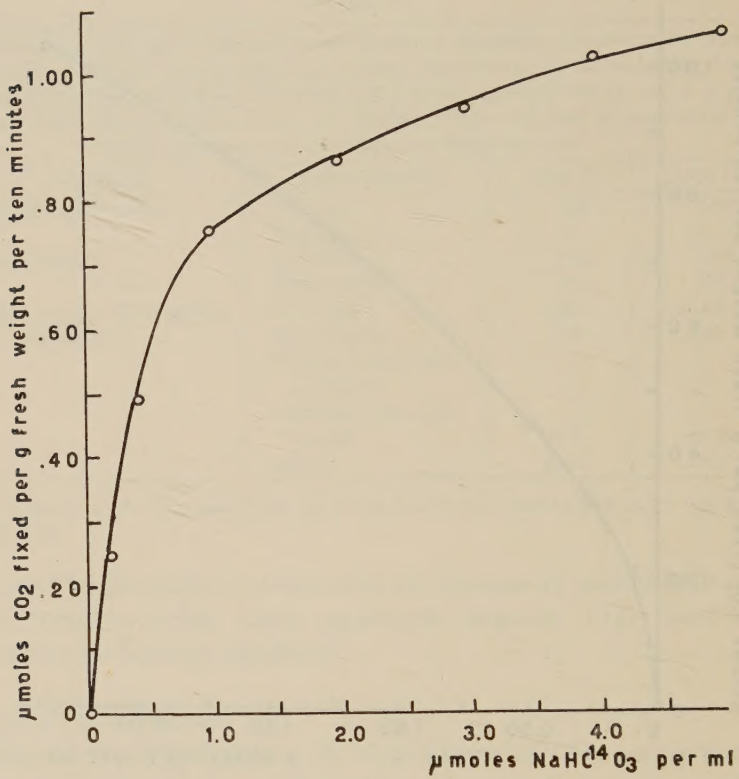

Fig. 3. Effect of HCO_3 concentration on CO_2 fixation by snap-bean root extracts. With the exception of the $NaHC^{14}O_3$ variables the standard reaction mixture (Figure 1) was used. Reaction time, 10 minutes.

TABLE 5

Influence of fluoride on CO_2 fixation by bean-root extracts when 3-PGA and PEP are employed as substrates. Standard reaction mixture. Reaction time, 10 minutes

Treatment	μmoles CO_2 fixed per g fresh weight	
	No KF added	5.0 μmoles KF per ml
No substrate	.003	—
PEP	.547	.505
3-PGA	.120	.018

Metal requirements

Although a Mg requirement for CO_2 fixation was not evident in experiments using non-dialyzed extracts, activity was increased by addition of Mg when dialyzed preparations were used. Table 6

TABLE 6

Influence of Mg and Mn on activity of PEP carboxylase of bean root. Extracts dialyzed 5 hours against 0.01 *M* Tris buffer pH 7.0. Standard reaction mixture except for divalent cation variables. Reaction time, 10 minutes.

Salts added (16 μmoles/ml)	μmoles CO_2 fixed per g fresh wt.
None	.039
$MgCL_2$	.123
$MnCl_2$	.022
$MgCl_2 + MnCl_2$	.021

presents results of an experiment using bean-root extracts dialyzed for 5 hours against 0.01*M* Tris buffer pH 7.0. Although considerable activity was lost by dialysis, 16 μmoles of $MgCl_2$ resulted in a definite enhancement of activity. Mn could not substitute for Mg and was inhibitory at this concentration. With wheat germ [24] and leaves of

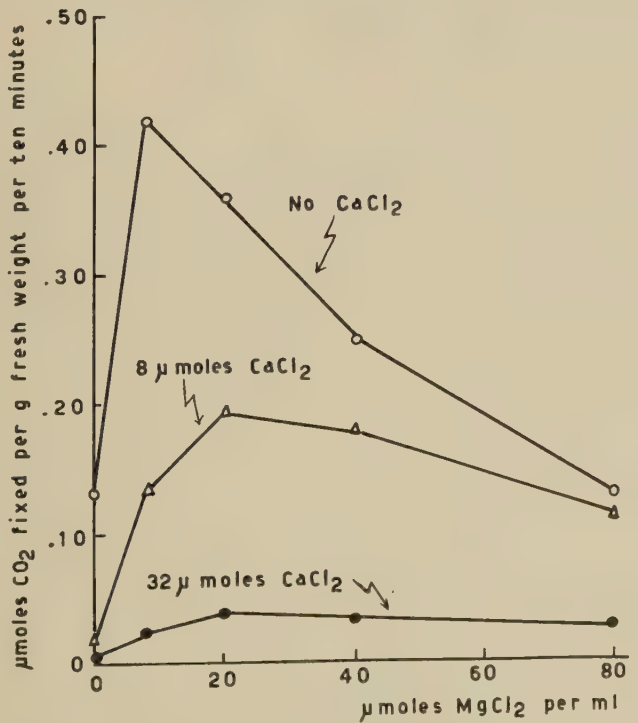

Fig. 4. Influence of Mg and Ca upon PEP carboxylase of snap-bean root. With the exception of Ca and Mg variables the standard reaction mixture (Figure 1) was used. Root extract dialyzed 4 hours against 0.01 *M* Tris buffer pH 7.4. Reaction time, 10 minutes.

succulents [30], Mn at somewhat lower concentrations was found to stimulate activity of PEP carboxylase.

Calcium also inhibited the reaction (Figure 4). In the absence of added Ca, the activity of the dialyzed preparations increased 225 per cent by the addition of 8 μmoles $MgCl_2$ per ml, but further increments resulted in progressive reductions in the rate of CO_2 fixation. The addition of 8 μmoles $CaCl_2$ per ml almost completely prevented CO_2 fixation in the absence of added Mg and this Ca inhibition could not be completely overcome by additions of Mg to the system.

Experiments with non-dialyzed extracts showed no consistent change in activity when KCl was varied from 0 to 96 μmoles per ml. Dialysis and use of relatively low $NaHCO_3$ concentrations also failed to produce a response to KCl (Table 7). Under these conditions, KCl at 64 μmoles per ml had little effect on activity with either PEP or 3-PGA as substrate.

TABLE 7

Influence of KCl on PEP and 3-PGA enhanced CO_2 fixation by bean-root extracts. Standard reaction mixture except for use of 0.40 μmoles $NaHCO_3$ per ml. Extract dialyzed 4 hours against 0.01 *M* Tris buffer pH 7.0. Reaction time, 10 minutes

Substrate (2.33 μmoles per ml)	μmoles CO_2 fixed per g fresh wt.	
	No KCl	64 μmoles KCl per ml
None	.000	.000
PEP	.258	.219
3-PGA	.040	.038

Products of the reaction

In order to determine the end products of CO_2 fixation under the conditions described here, aliquots of the reaction mixture shown in Table 3 were desalted by repeated extraction with acidified acetone [19]. The extracted material was passed through a strong H-resin and chromatographs of the material passing through, containing organic acids among other components, were developed with butanol/formic acid/H_2O (10 : 2 : 15). The amino acids retained on the resin were eluted with NH_4OH and chromatograms developed using butanol/acetic acid/H_2O (25 : 6 : 25). Organic-acid spots were developed with chlorophenol red and amino acids with ninhydrin [4]. A small amount of malate and aspartate were added to the respective solutions and radioactivity associated with the spots developed by

these compounds was determined by counting the spots with a windowless flow counter. One-dimensional chromatography did not permit separation of aspartate and glutamate, so these amino acids are considered together in the presentation of the data. In the reaction mixtures, the root extracts of which were prepared after previously soaking the roots in 1 per cent NH_4OH, 48 per cent of the fixed $C^{14}O_2$ was recovered in aspartate and/or glutamate, while considerably smaller amounts were found in malate (Table 3). However, further experiments (Table 8) of shorter duration show that, in the absence of NH_4, malate accounted for 64.2 per cent

TABLE 8

Incorporation of radioactivity into malate and aspartate and/or glutamate by bean-root extracts as influenced by NH_4OH pretreatment. Standard reaction mixture in total volume of 2.3 ml. Reaction time, 10 minutes

Root treatment prior to enzyme extraction	μmoles CO_2 fixed per g fresh wt.	Percental of total fixed radioactivity in:	
		Malate	Aspartate and/or glutamate
15 min. in 1% NH_4OH	1.06	11.5	28.9
15 min. in H_2O	1.20	64.2	14.1

of the total radioactivity after 10 minutes reaction, whereas it accounted for only 11.5 per cent when roots had been pretreated with NH_4. On the other hand, 28.9 per cent of the activity was found in aspartate and/or glutamate after NH_4 pretreatment and only 14.1 per cent was found in these compounds in the absence of NH_4. In spite of the large difference in the direction of the reaction, the total amount of CO_2 fixed was not greatly influenced by the NH_4 pretreatment.

General occurrence of PEP carboxylase in plant tissue

In view of the probable importance of carboxylation of PEP as a mechanism of entry of CO_2 into the Krebs cycle components or amino acids and thus its possible influence on protein synthesis as well as carbohydrate metabolism, a survey of numerous species and plant tissues was undertaken. The assay was as described previously, except that for chlorophyll-containing tissue the reaction was carried out in the dark.

The data are presented in Table 9 and illustrate the presence of

TABLE 9

Phosphoenolpyruvate carboxylase activity of various tissues. All extracts prepared by grinding 15 g fresh tissue with 10 ml 0.1 *M* Tris buffer pH 7.4. Standard reaction mixture. Reaction time, 10 minutes

Species	Tissue	μmoles CO_2 fixed per g fresh wt.	
		No PEP	2.38 μmoles PEP per ml
Alfalfa	Leaf blades	.042	1.339
Cabbage	Leaves	.010	1.351
Carrot	Roots	.013	.481
Corn	Leaves	.026	.949
Cotton	Leaf blades	.006	.657
	Roots	.000	.237
Irish potato	Leaf blades	.025	1.114
	Tubers	.028	.809
Peanut	Leaf blades	.013	.568
Snap bean	Leaf blades	.006	.860
	Stem	.006	.260
	Seed	.003	.719
	Roots	.000	.527
Sweet potato	Leaf blades	.015	.387
	Roots	.009	.886
Tobacco	Leaves	.004	.682
Tomato	Leaf blades	.013	1.324
Watermelon	Leaf blades	.046	1.859
	Roots	.006	.610

the enzyme system in all tissues examined. Strong systems were found in snap-bean leaves, stems and seed, as well as in roots. The storage tissue examined showed considerable activity, as did the fibrous roots of watermelon and cotton. Leaves of all species contained very active PEP carboxylase.

As the plants were not of comparable age, direct comparisons of activity between species cannot be made. The data, however, emphasize the general occurrence of this enzyme. With some tissues, small but appreciable amounts of CO_2 were fixed in the absence of added PEP. Although in these cases other CO_2-fixing mechanisms may be operative, an alternative explanation would be the presence of a native supply of PEP being available for action by PEP carboxylase.

DISCUSSION

There are a number of possible reactions through which CO_2 may be fixed non-photosynthetically by plant tissue. Two enzymes, malic enzyme [7] and oxaloacetic decarboxylase [9], have been shown

to directly carboxylate pyruvate. However, the equilibrium of these reactions highly favors decarboxylation. Furthermore, these are not applicable to the bean-root preparations reported here, as neither acetate nor pyruvate served as substrates for CO_2 fixation (Table 4). Fluoride inhibition of CO_2 fixation when 3-PGA served as substrate and its failure to inhibit fixation when PEP was the substrate (Table 5) indicate that pyruvate was not carboxylated directly. The close agreement of the 1 : 1 molar ratio of inorganic phosphorus produced to CO_2 fixed further supports the operation of reaction *a* in these investigations.

The enhanced oxidation of DPNH with the addition of HCO_3 at pH 7 (Tables 1, 2) may be considered as indirect evidence for the formation of oxaloacetate from PEP. Since the equilibrium for malic dehydrogenase is strongly in favor of reduction of oxaloacetate to malate by DPNH [6], the formation of oxaloacetate from PEP would result in increased oxidation of DPNH. Use of the coupled reactions has been used for assaying PEP carboxylase activity in wheat germ extracts [24] and in spinach leaves [1].

Although the rate of CO_2 incorporation was not greatly reduced when root tissue had been equilibrated previously with NH_4OH (Table 8), the majority of the labelled carbon was recovered in amino acids rather than in malate. Even though malate accumulation has been shown to be the major result of CO_2 fixation by intact root tissue [12 18], Jackson and Coleman [11] have shown this to be true in intact bean roots only in the absence of NH_4 in the ambient solution. Consequently, a mechanism capable of shuttling the fixed carbon to amino acids in the presence of NH_4 must be present in the bean roots. Similar results were obtained by Saltman *et al.*[20] in their investigation of dark fixation by leaves of succulents. As postulated by these investigators, a transamination involving oxaloacetate and glutamate (produced from α-keto-glutarate under the influence of large excess of DPNH and NH_4) would result in the accumulation of CO_2 in aspartate and glutamate. Such a scheme would allow a mechanism for incorporation of CO_2 into root proteins without first accumulating in soluble carbohydrates.

Magnesium was found to be required for PEP enhanced CO_2 fixation (Table 6, Figure 4). Tchen and Vennesland [24] indicate that the Mg effect is on oxaloacetate formation rather than on its reduction by DPNH. The reciprocal effects of Ca and Mg on CO_2

fixation by PEP carboxylase are similar to the effects of these two ions on conversion of PEP to pyruvate [16] and suggest a similarity in the modes of action of the two enzymes on the common substrate.

Although Graf and Aronoff [10] reported CO_2 fixation by intact soybean roots to be stimulated by KCl, no evidence for K stimulation of PEP carboxylase was observed in these experiments. Subsequent experiments * have shown that PEP carboxylase activity of K-deficient sweet-potato leaves is less than that of plants grown with adequate K. However, addition of KCl to the assay medium could not increase activity with either the K-deficient or K-adequate tissue, indicating a possible function of K in synthesis of the enzyme.

The large buildup of malate contents of plant roots which may result from the operation of PEP carboxylase, may have important implications so far as ion accumulation is concerned. The depressing effect of high pH on the absorption of anions [17] may be due to the production of "native" anions by this mechanism. Burstrom [3] has pointed out malate accumulation occurred when cation absorption exceeded anion absorption from external solutions. Jacobson and Ordin [13] confirmed these findings and Jacobson [12] found that the malate increase was a result of CO_2 fixation. Jackson and Coleman [11] obtained similar results and also noted that Ca absorption, as well as K absorption, was enhanced whenever conditions were favorable for malate accumulation. It appears that not only carbohydrate and nitrogen metabolism may be altered by operation of this system, but that important effects on ion absorption mechanisms may also result.

SUMMARY

Extracts of snap-bean roots are capable of fixing large amounts of CO_2 through carboxylation of phosphoenolpyruvate. Fixation was dependent upon a supply of PEP or substrate capable of being transformed to PEP. 3-PGA served as substrate, but when its conversion to PEP was blocked by fluoride, CO_2 fixation was eliminated. Acetate or pyruvate were not effective as substrates. The amount of CO_2 fixed was closely associated with production of inorganic phosphorus. From these characteristics and the unfavorable equilibria of the other known carboxylating reactions associated with the

* G. W. Thomas, N. T. Coleman and W. A. Jackson. Unpublished data, N. C. State College, 1957.

Krebs cycle, it is concluded that PEP carboxylase accounts for the major entrance of CO_2 into the Krebs cycle.

Magnesium stimulated the reaction, while Ca inhibited it. Potassium additions to the assay mixture were found to have no direct effect on stimulation of CO_2 fixation. In the presence of NH_4, amino acids rather than malate were found to contain the majority of the added CO_2.

Some implications of the importance of this enzyme system in metabolism of the root and on the soil-root relationship are discussed.

Received July 22, 1958

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INTERACTIONS IN THE POTASSIUM AND SODIUM ABSORPTION BY INTACT MAIZE SEEDLINGS

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INTRODUCTION

Selective absorption of potassium and sodium by plant tissue is a well-known fact, but its mechanism is still a matter of discussion.

As pointed out by Sutcliffe [11 12] in the light of the carrier hypothesis there are two ways in which such a selectivity might be explained:

I. There is one type of carrier for each ion or group of ions. In this case selectivity depends on the relative number and rate of transport of the respective carriers.

II. There is only one type of carrier for all ions. In this case selectivity is based on preferential adsorption and/or transport of special ions.

Epstein and Hagen [2] claim to have shown that there is no direct competition in the uptake of Rb and Na by excised barley roots (except at high concentrations). So they conclude that there are different binding sites for Rb and K on the one hand and for Na on the other. Scott and Hayward [10] arrive at essentially the same conclusion for the marine alga *Ulva lactuca*.

On the other hand it is the opinion of Jacobson *et al.*[6] working with excised barley roots that there is probably a direct competition between K and Na for the same binding site. Also Sutcliffe [11 12] concludes from his experiments on the intake of alkali cations by storage tissues (red beet, potato, swede) and barley roots (excised and non-excised) that there is only one type of ion carrier

able to transport all kinds of alkali cations but preferring a special kind if a choice is available. This preference differs for different plant species.

Schuffelen [9] seems to take an intermediate position between these extremes. In addition to a general adsorption surface on the root from which the ions are removed metabolically he considers that there is possibly a special K-carrier.

In view of these conflicting opinions the present investigation was designed to throw more light upon the mechanism of the selective absorption of K and Na.

MATERIAL AND METHODS

a. *Plant material*

Maize (single cross D × 9) was chosen as a test plant. All plants used in the experiments were raised from one batch of seed (harvest 1956) obtained from the College of Agriculture, University of Wisconsin, Madison, U.S.A..

In order to obtain comparable plant material during the whole of the experimental work sets of 25 seedlings were raised in a strictly constant way in an air-conditioned room (temperature 25°C, relative humidity 70%) as follows:

About 150 seeds were soaked in aerated distilled water for 24 hours and then allowed to germinate in the dark in three groups of 50 seeds each on a layer of wet cotton-wool between two sheets of filter paper. After 66 hours 25 seedlings were selected from each group and mounted on a zinc grid coated with paraffin wax (see Fig. 1, a and b) with drops of a low-melting mixture of beeswax and vaseline. The three grids were then placed on 800-ml glass beakers filled with about 500 ml of $10^{-4}M$ $CaSO_4$ solution so that the level of the solution was just below the seeds.

These experimental units were placed in an artificial light chamber containing a high-pressure mercury lamp (Philips HO 2000, 450 W) and four incandescent lamps (Philips Comptalux, 150 W). Between the lamps and the plants (distance about 45 cm) was a layer of flowing tap water of about 1 cm thickness. All units were aerated continuously.

The $CaSO_4$ solution was renewed every 24 hours except during the week-ends. When the seedlings were 13 days old, the $CaSO_4$ solution was replaced by a dilute culture solution for 24 hours. For this purpose the Woodford and Gregory [13] culture solution was used with the addition of micronutrients (1 ml Hoagland A–Z solution per litre culture solution). When the plants were 20 days old they were used in the experiment. Each set of plants was used only once.

The average fresh weight of the roots of one set of plants amounted to about 15 grams.

Seedlings raised in this way do not show any marked deficiency symptoms though they are typical low salt plants.

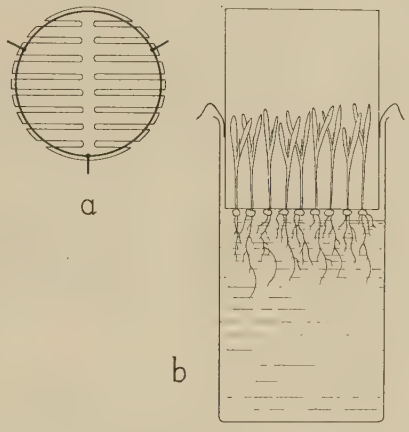


Fig. 1. a. Zinc grid used for the mounting of seedlings (topview). – b. Grid with seedlings on a 800-ml glass beaker.

b. *Experimental technique*

The continuous-flow technique as introduced by van den Honert [4] (see also Becking [1]) was used throughout the experiments. For details of this technique may be referred to these papers. Its principle is that a salt solution is flowing through the root vessel at a constant rate. Absorption by the roots causes the concentration of ions to be lower in the discharged then in the supplied solution. After the establishment of a steady state the rate of uptake can be calculated from the formula:

rate of uptake (me/h)	=	*rate of inflow* (l/h)	×	*concentration of supplied solution* (me/l)	—	*rate of outflow* (l/h)	×	*concentration of discharged solution.* (me/l)

In the experiments of van den Honert and Becking the absorption rate under certain standard conditions was taken as a base of reference. However, this design had to be modified because under the conditions described the rate of growth of the seedlings was too low to ensure a prolonged high rate of uptake of K and Na. Therefore the procedure was as follows:

The three sets of plants were transferred to the apparatus at 5.00 p.m. after the $CaSO_4$ solution had been replaced by about 500 ml of distilled water. The rate of flow was 100 ml/h throughout. If no ions were absorbed it would take 17.5 hours for the concentration in the vessel to become 97% of the concentration of the supplied solution (cf. Becking [1], p. 18/19). The absorption by the plant accelerates the establishment of a steady state, so the next morning at 10.00 a.m. samples could be taken. Control measure-

ments in samples taken a few hours later did not reveal deviations from the steady-state condition exceeding the limits of accuracy of the experimental technique (see Table 1).

TABLE 1

Establishment of the steady-state condition as revealed by successive samplings						
K-concentration of supplied solution (me/l)	K-concentration of discharged solution (me/l)		Transpiration ml/h		Rate of K-absorption me/h	
	1 *	2 *	1	2	1	2
1.987	1.585	1.590	1.56	1.92	0.0422	0.0423
1.281	1.038	1.051	1.34	1.66	0.0254	0.0245
0.632	0.422	0.422	1.86	2.23	0.0218	0.0219

* 1 = after 17 hours.
2 = after 21 hours.

Two air tubes per root vessel were used ensuring sufficient aeration and stirring of the solutions. However, in the experiments at very low potassium concentrations, when the rate of uptake is still high, very efficient stirring is necessary. In this case a special device was used to supply the solution just above the outlet of the air tubes so that a rapid spreading over the whole root vessel was ensured.

The discharged solutions were sampled in 50-ml graduated flasks and the time needed for filling them was measured with a stopwatch. After the pH in the root vessels had been determined with a glass electrode the supplied solutions were sampled in the same way. The rate of outflow was only a little lower than the rate of inflow as the transpiration of the plants usually did not exceed 2 ml/h.

During the experiments the plants were exposed to a high-pressure mercury lamp (Philips HO 2000) at a distance of about 70 cm.

The root vessels were placed in a water thermostat maintained at 20°C.

c. *Solutions*

The K- and Na-ions were supplied as orthophosphates throughout.

Primary and secondary phosphate were mixed in the ratio necessary to bring the pH in the root vessel near 6. In fact, the average pH in the experiments was 5.9. If the pH was lower than 5.4 or higher than 6.4 the experiment was discarded, but in most cases deviations did not exceed 0.3 pH unit.

At low K-concentrations in the absence of Na, when the rate of K-uptake is still high, it appeared to be necessary to add some triethylamine to the solutions for the adjustment of the pH.

d. *Estimation of K and Na*

A Beckman flame photometer was used for the determination of the K- and Na-content of the supplied and discharged solutions.

Linear interpolations in a wide series of standard solutions of KCl and

NaCl were applied. To avoid interference errors the K-(Na-)standards were given about the same Na-(K-)content as the solutions to be analysed.

EXPERIMENTAL RESULTS

Four series of experiments were run to establish the following relationships:

1. Na-absorption in relation to the Na-concentration in the absence of K.
2. Na- and K-absorption in relation to the Na-concentration at a constant K-concentration.
3. K-absorption in relation to the K-concentration in the absence of Na.
4. K-absorption in relation to the K-concentration in the presence of a constant high Na-concentration.

Series 1: Na-absorption in the absence of K (see Fig. 2)

The rate of Na-uptake appears to depend on the Na-concentration around the roots in the familiar way: a rapid increase at low concentrations gradually passes into practical independence at higher concentrations.

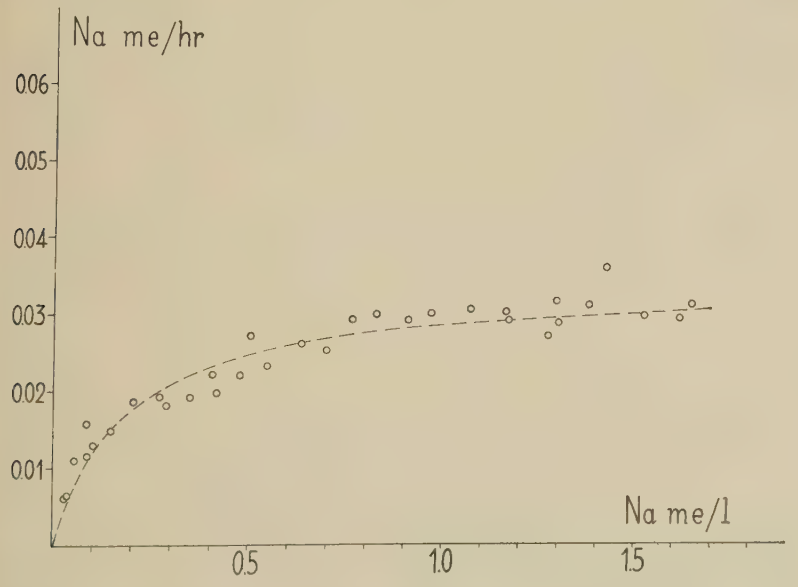


Fig. 2. Na-absorption in relation to Na-concentration in the absence of K.

Series 2: Na- and K-absorption in the presence of a constant K-concentration (see Fig. 3)

Essential changes occur in the presence of a constant concentration (about 1.6 me/l) of K. A strong inhibition of the Na-absorption is apparent which is largest at the lowest concentrations and gradually decreases at higher concentrations. At the same time the K-absorption is gradually inhibited by increasing amounts of Na.

At a concentration of 1.6 me/l the rate of K-absorption in the absence of Na appears to be nearly twice as large as the rate of Na-absorption in the absence of K (cf. Figs 2 and 3).

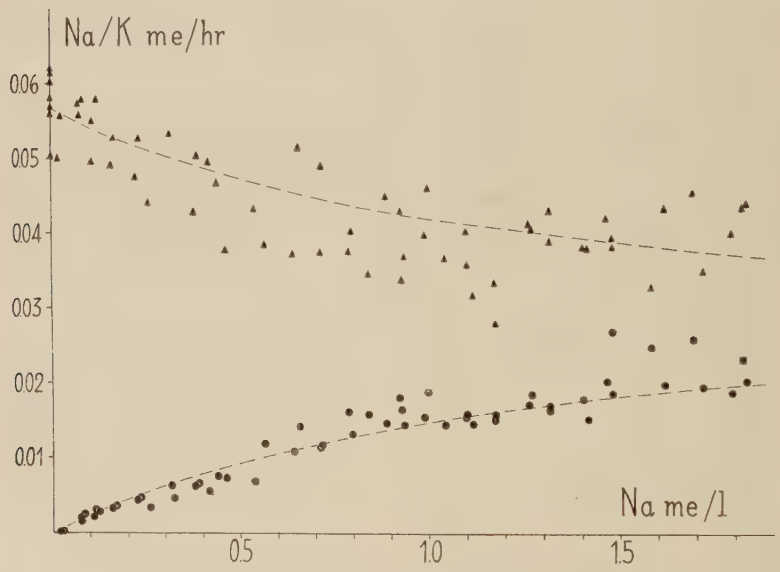
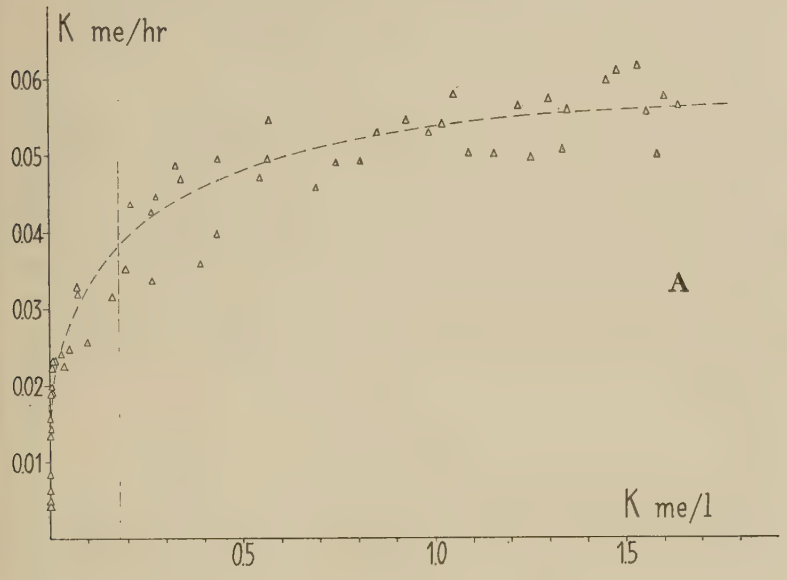


Fig. 3. Na- and K-absorption in relation to Na-concentration at a constant K-concentration of about 1.6 me/l.

Series 3: K-absorption in the absence of Na (see Fig. 4, a and b)

The relation between the rate of K-absorption and K-concentration in the absence of Na is strikingly different from what was found if only Na was supplied (cf. Fig. 2). Here there is an extremely steep rise of the absorption rate at the lowest concentrations up to a certain level (about 0.02 me/h). Starting from this level the relation shows the familiar features again.



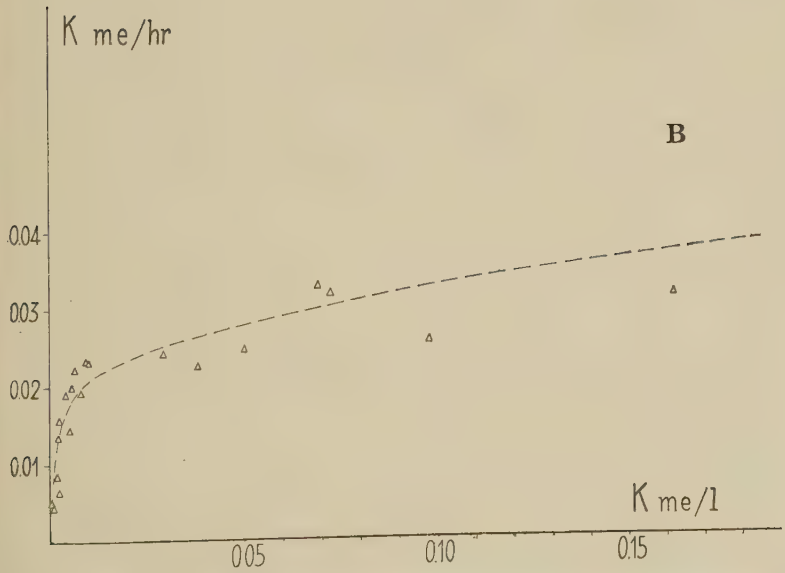


Fig. 4. *a*. K-absorption in relation to K-concentration in the absence of Na. *b*. The same as *a* but with the concentration put on a larger scale to show the initial part in detail.

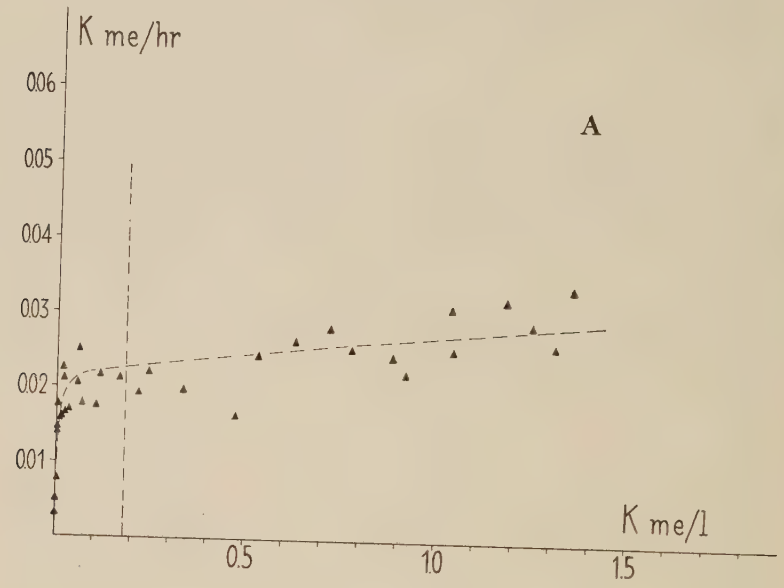


Fig. 5. *a*. K-absorption in relation to K-concentration at a constant Na-concentration of about 4.85 me/l. – *b*. The same as *a* but with the concentration put on a larger scale to show the initial part in detail.

Series 4: K-absorption in the presence of a constant Na-concentration (see Fig. 5, a and b)

A high degree of inhibition of the K-absorption is apparent in the presence of about 4.85 me/l Na. However, this inhibition does not apply to the initial steep part at the lowest concentrations which remains largely unchanged. Only the part above the aforementioned absorption level of about 0.02 me/h is strongly affected and transformed into a very slowly rising straight line.

The Na-absorption could not be measured in this series the amount absorbed being too small a fraction of the total amount present.

DISCUSSION

It is clear from the results mentioned in the preceding section that at all concentrations the plants used in these experiments prefer K to Na if the ions are supplied separately. This preference is specially marked at low concentrations. For instance, from a concentration of 0.01 me/l K about ten times as much K is absorbed as Na from an equivalent Na-solution. If both ions are supplied together in a concentration of 1.6 me/l the rate of absorption is about twice as large for K as it is for Na (see Fig. 3). So our plants show a marked selectivity.

However, in this case selectivity could not be based on a system of specific and strictly separate carriers (case I of the introduction) as postulated by Epstein and Hagen [2], for competition phenomena are evident.

On the other hand a one carrier system (case II of the introduction) as postulated by Sutcliffe [11 12] is precluded by the fact that part of the mechanism of K-absorption is extremely insensitive to even a large excess of Na.

The only obvious explanation which does not violate the carrier hypothesis seems to be that here two carriers are involved in the K-absorption:

1. a *carrier A* that is able to transport K only and for which Na shows hardly, if any, affinity.
2. a *carrier B* that is able to transport both K and Na and for which these ions compete if supplied together.

A quantitative treatment of the experimental results strongly supports this view.

According to the carrier theory of ion absorption the free ions combine with carrier molecules in a reversible reaction:

$$\text{ion} + \text{carrier} \underset{k_1}{\overset{k_2}{\rightleftharpoons}} \text{ion-carrier complex.}$$

The ion-carrier complex is thought to pass through a membrane impermeable to the free ions and then to be broken down metabolically in an irreversible reaction in which the bound ions are released again:

$$\text{ion-carrier complex} \xrightarrow{k_3} \text{carrier}' + \text{ion.}$$

The kinetics of this reaction scheme are expressed by an analogue of the Michaelis-Menten equation for enzyme reactions:

$$v = \frac{c.V}{K + c}$$

in which v = rate of uptake of ion (me/h), c = concentration of ion (me/l), V = maximal rate of uptake of ion (me/h) and $K = (k_2 + k_3)/k_1$ = Michaelis-Menten constant = concentration at which $v = \frac{1}{2}V$ (me/l).

The data on Na-absorption in the absence of K (Fig. 2) fit this equation well (see dotted line). The computed values of V and K are 0.0336 (me/h) and 0.180 (me/l) respectively.

On the contrary the data on K-absorption in the absence of Na (Fig. 4, a and b) do not fit the equation which demonstrates the complex character of this relationship. If, indeed, this complexity is due to the fact that two carriers are involved in the K-uptake, the data must answer the equation:

$$v = \frac{c.V_A}{K_A + c} + \frac{c.V_B}{K_B + c}$$

in which V_A and K_A are the characteristics of carrier A and V_B and K_B of carrier B.

A good fit is obtained with the following values of the constants (dotted lines in Fig. 4, a and b):

$V_A = 0.0220$ (me/h) $\qquad K_A = 0.0015$ (me/l)
$V_B = 0.0402$ (me/h) $\qquad K_B = 0.257$ (me/l).

Carrier B was supposed to be identical with the carrier transporting Na whereas the K-transport by carrier A was thought to be

highly unaffected by Na. With the values of the respective constants known it should be possible to check this hypothesis by computing the competition curves to be expected theoretically if K and Na are supplied together.

The rate of absorption of one ion (index 1) in the presence of an other ion (index 2) competing for the same binding site is given by the equation (cf. Epstein and Hagen [2]):

$$v_1 = \frac{c_1 V_1}{K_1 + c_1 + (K_1/K_2).c_2}.$$

The dotted lines in Figs. 3 and 5 (a and b) represent the computed competition curves. It is seen that there is a considerable agreement with the experimental results.

The specificity of carrier A may not be absolute. In fact, in the absence of Na the concentration at which the K-transport by this carrier equals $\frac{1}{2}V_A$ may be somewhat lower then in the presence of 4.85 me/l Na. This is apparent from Figs. 4b and 5b in which K-values of 0.0015 and 0.0030 respectively were used to obtain the best fit. However, it should be emphasized that this difference may not be significant, taking into account the limits of accuracy at these low concentrations. In any case it does not detract a great deal from the extremely high specificity of carrier A.

Attention should be drawn to another circumstance. The maximal rate of absorption by carrier B appears to be larger for K (0.0402 me/h) than for Na (0.0336 me/h) and the same is the case for the Michaelis-Menten constants (0.257 and 0.180 me/l respectively). In terms of the carrier theory this may be readily interpreted as the result of a higher rate of metabolic breakdown of the ion-carrier complex (or in other words a higher value of k_3) in case of the weaker binding with K. However, if not the rate of breakdown of the ion-carrier complex but its rate of transport through a plasmatic boundary should limit the absorption rate, the difference between the values of V_B for K and Na might be explained (van den Honert [5]) by the assumption that the ion-carrier complex passes through this boundary more easily if charged with the less hydrated K-ion.

CONCLUSION

In the light of the conflicting opinions about the mechanism of the selective absorption of K and Na as outlined in the introduction these results are of some interest as they show that both mechanism I and II may operate at the same time. In the present case selectivity is mainly due to mechanism I (the highly selective carrier A for K-absorption), mechanism II (carrier B transporting both K and Na) showing little preference for the one ion or the other.

That more than one carrier might be operative in K-absorption was also concluded by Kahn and Hanson [8] from experiments on the kinetics of K-absorption in maize seedlings as related to cell maturity. These kinetics appeared to differ for different root zones. They suggest that more than one site might be involved in K-absorption and that the ratio of the activities of these sites might be a function of cell maturity. In fact, an unequal distribution of the activities of carrier A and B over the root length may have prevailed in the plant material used in the experiments described above.

Binding of K and Na by more than one site is also postulated by Fried and Noggle [3] for excised barley roots. However, these authors did not look into the possibility of identifying K- and Na-carriers.

Of course it would not be justified to generalize from the presented conclusions. In other plant material conditions may be quite different and even in the same material the situation may depend upon such factors as age, nutritional history, growth, *etc.* How cautious one should be in this respect was emphasized by Jacobson *et al.* and is also demonstrated by the varying results obtained with the same object (*i.c.* excised barley roots) by different authors (Jacobson *et al.*[6], Epstein and Hagen [2], Sutcliffe [11], Fried and Noggle [3]).

SUMMARY

The relation between the rate of K- and Na-absorption by maize seedlings in water culture and the concentration of these elements in the solution was investigated in a series of experiments.

Na-absorption appeared to be strongly inhibited by excess K, but only part of the K-absorption was inhibited by excess Na.

These results are interpreted in terms of the carrier hypothesis. Two carriers are thought to be operative:

1. a highly selective carrier A transporting K only and hardly affected by excess Na (Michaelis-Menten constant = ab. 0.002 me/l);
2. a hardly selective carrier B transporting both K and Na (Michaelis-Menten constant = ab. 0.2 me/l).

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COMPETITION FOR NUTRIENTS BETWEEN GRASSES AND WHITE CLOVER

I. EFFECT OF GRASS SPECIES AND NITROGEN SUPPLY

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INTRODUCTION

In grass–clover associations, interspecific competition for nutrients is apparently a considerable factor in determining clover production. This has been well demonstrated under conditions of low potassium (Blaser and Brady [1], Brown and Rouse [2]), phosphorus (Sears [3], Trumble and Shapter [4], Walker *et al.*[7]) and sulphur (Walker and Adams [5]) supply.

The available nitrogen supply has a remarkable effect on the grass–clover balance. While this can frequently be explained in terms of increasing competition for light by nitrogen treated grass, there is evidence to suggest a direct increase in nutrient competition by grasses with increase in nitrogen supply (Blaser and Brady [1]). This may be a consequence of observations by Walker *et al.*[6] that grasses take up 95 per cent of the mineral nitrogen utilised by a grass-clover association, and increasing the supply of combined nitrogen will thus automatically intensify the competition offered by grasses for other nutrients.

It is well recognised that grasses differ in their ability to compete with white clover and this is a factor of importance in compounding pasture mixtures. The ability of some New Zealand pasture grasses to compete with white clover for nutrients was therefore tested and the effect of increasing nitrogen status on nutrient competition examined.

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MATERIALS AND METHODS

Pot trials were laid out to study competition for a nutrient anion (phosphate) and a nutrient cation (potassium) by each of three grasses – short-rotation ryegrass, cocksfoot, and browntop – with white clover. The phosphate competition trial was a $3 \times 3 \times 3 \times 2$ confounded factorial with two replications, the factors being (1) three competing grass species (2) three nitrogen treatments (applied as NH_4NO_3): No applied N(N_0), N applied at 200 lb. per acre (N_1) and N at 400 lb. per acre (N_2), (3) three phosphate treatments (applied as $Ca(H_2PO_4)_2$): No applied P (P_0), P at 40 lb. P_2O_5 per acre (P_1) and P at 80 lb. P_2O_5 per acre (P_2), and (4) two cutting treatments: Uncut (C_0) and cut when light interference between species was obvious (C_1). In conjunction with this trial, another factorial (4×2 with 2 reps.) was studied in order to observe the phosphate uptake of the species when unassociated at two phosphate levels, the P_0 and P_2 treatments of the main trial.

The experiments were conducted in five-inch plastic pots each containing 800 g (air dry) of a phosphate-deficient (Truog P 1.3 mg% P_2O_5) Templeton silt loam. Seed was sown in the pots at equidistant spacings by means of a punched card and, in the pots containing the associations, the grass and clover were alternately spaced. Pots were finally harvested at the time of the third cut of associations receiving the cutting treatment. All cuts were bulked for analysis of dry-matter production and phosphorus uptake of the grass and clover per pot.

The potassium competition trial was carried out in similar pots containing 450 g of potassium-deficient Horotiu sandy loam (exchangeable K 0.21 me%) mixed with 320 g of sand. The experiment consisted of two factorial trials, a $3 \times 3 \times 3$ testing the competitive effects of the associated grass on white clover and a $4 \times 3 \times 3$ (—6) studying the four species grown separately. The competing grass species were as in the phosphate experiment. Three N levels were applied as in the phosphate trial, but in this experiment, the nitrogen application was repeated after the second harvest. When grown alone, the clover pots did not receive the N_1 and N_2 treatments. Three potassium levels were studied: No applied K (K_0), 100 lb. K_2O as KCl per acre (K_1) and 200 lb. K_2O as KCl per acre (K_2).

The herbage from all pots was harvested three times over the growing period at a stage of growth designed to reduce the effect of light competition between the associated species. The cuts were bulked for analysis of dry matter and potassium uptake by the grass and clover per pot.

RESULTS

A. *Competition for phosphate*

Average dry-matter yields of clover grown alone and in association are shown in Figure 1. There was a highly significant ($F = 14.18$

at d.f. 2, 49) and linear depression of clover yield with nitrogen application when grown in association with grass but, as there was no interaction between this and any other variant, the results of the N treatments have been bulked in the figure. The association of grass caused a highly significant reduction in clover yield ($F = 38.14$ at d.f. 1, 71) and there were highly significant differences in these reductions of clover yield among the different grass associations ($F = 402.17$ at d.f. 2, 49).

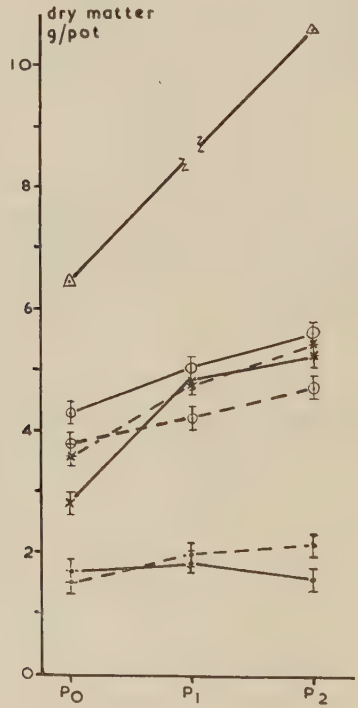

Fig. 1. Effect of P treatment on dry-matter yield of clover grown alone and in association. Average of 3 N levels.

——— Uncut △ Grown alone ⊙ With cocksfoot
——— Cut × With browntop • With ryegrass

The average yield of clover over all treatments in the different associations was inversely related to the yield of the associated grass.

In a similar manner, the reduction of clover yield due to nitrogen application may be attributable to increased competition from the grasses due to the response of the latter to nitrogen.

Phosphate application markedly increased the clover yield whether grown alone ($F = 14.27$ at d.f. 1,71) or in association ($F = 55.36$ at d.f. 2,49) but cutting had no significant effect on clover yields of the associations. However, when clover yields are expressed in proportion to the total yield, there is an overall increase in percent clover per pot due to cutting.

The differential response of clover to phosphate applications both between clover grown alone and in association, and among the different grass–clover associations, noted in the highly significant G × P interaction ($F = 10.4$ at d.f. 1,71 for alone v. associated and $F = 13.9$ at d.f. 4,49 among associations), may be observed in the response slopes to P in Figure 1. The figure also illustrates the highly significant grass × cutting interaction on clover yield ($F = 10.23$ at d.f. 2, 49).

The percent phosphorus of grass and clover is shown in Table 1. The overall response to phosphorus plus the fact that phosphorus application gave little or no increase in percent P of grass or clover in uncut associations, indicate that phosphorus deficiency was limiting the production of all species in these pots. In the cut associations, increasing phosphate application from P_0 to P_2 resulted in an increase in the average P content of clover from 0.10 to 0.13 per cent when associated with ryegrass and cocksfoot, but only from 0.10 to 0.11 per cent when with browntop. A similar increase in P content with increasing phosphate application to the cut associations was noted in browntop (0.11 to 0.13 per cent) and in ryegrass (0.09 to 0.11 per cent) but a smaller increase was observed in cocksfoot (0.09 to 0.10 per cent).

Phosphorus uptake data (Table 2) show responses to N and P treatments similar to those observed in dry-matter yield.

B. *Competition for potassium*

Average dry-matter yields of clover grown alone and in association are shown in Figure 2. Again the N treatments have been bulked because, although there was a significant ($F = 5.1$ at d.f. 2, 25) linear reduction of clover yield with nitrogen application, the N effect was independent of the other variables. The reduction in

TABLE 1

Percent phosphorus (P) of oven-dried grass and clover								
Associated grass	Treatment (C and P)		Clover			Grass		
			N_0	N_1	N_2	N_0	N_1	N_2
Ryegarss	C_0	P_0	.082	.087	.075	.050	.044	.044
		P_1	.082	.079	.085	.048	.053	.047
		P_2	.081	.091	.085	.060	.052	.048
	C_1	P_0	.096	.106	.101	.092	.093	.094
		P_1	.120	.101	.113	.101	.099	.091
		P_2	.128	.135	.136	.111	.110	.111
Cocksfoot	C_0	P_0	.068	.068	.064	.034	.050	.037
		P_1	.071	.074	.071	.041	.050	.037
		P_2	.073	.077	.081	.036	.044	.047
	C_1	P_0	.101	.099	.111	.081	.099	.086
		P_1	.112	.113	.125	.083	.083	.131
		P_2	.123	.129	.130	.073	.103	.119
Browntop	C_0	P_0	.064	.066	.070	.053	.050	.046
		P_1	.063	.070	.066	.052	.070	.049
		P_2	.076	.086	.066	.054	.057	.047
	C_1	P_0	.117	.099	.095	.104	.116	.112
		P_1	.119	.108	.116	.116	.107	.125
		P_2	.112	.106	.113	.123	.131	.139
Clover (Unassoc.)	C_0	P_0	.074					
		P_2	.081					
Ryegrass (Unassoc.)	C_0	P_0						.046
		P_2						.058
Cocksfoot (Unassoc.)	C_0	P_0						.041
		P_2						.046
Browntop (Unassoc.)	C_0	P_0						.058
		P_2						.058

clover yield with nitrogen application could again be attributed to competition from N-responsive, associated grass. The effect of associated grass on clover yield was similar to that observed in the phosphate trial and was highly significant ($F = 102.6$ at d.f. 1, 53 for effect of association and $F = 41.6$ at d.f. 2, 25 among associations). There was a linear clover response to potassium ($F = 8.6$ at d.f. 2, 25).

The interaction between associated grass and potassium treatment ($F = 3.9$ at d.f. 4, 25) is seen to be due to the reduction of clover

TABLE 2

Phosphorus uptake (mg P × 100) of grass and clover per pot								
Associated grass	Treatment (C and P)		Clover			Grass		
			N_0	N_1	N_2	N_0	N_1	N_2
Ryegrass	C_0	P_0	160	123	120	408	401	383
		P_1	135	176	141	414	456	454
		P_2	177	127	99	505	617	620
	C_1	P_0	181	153	111	524	590	645
		P_1	281	195	171	545	723	768
		P_2	370	254	250	695	824	934
Cocksfoot	C_0	P_0	331	282	243	126	200	184
		P_1	382	382	322	151	231	243
		P_2	405	416	378	152	248	334
	C_1	P_0	461	341	352	205	341	387
		P_1	496	440	472	302	348	600
		P_2	556	736	569	314	409	606
Browntop	C_0	P_0	170	179	210	308	311	278
		P_1	316	343	300	237	277	263
		P_2	432	451	315	314	483	433
	C_1	P_0	468	359	307	337	477	520
		P_1	553	535	541	379	419	447
		P_2	628	570	637	357	554	642
Clover (Unassoc.)	C_0	P_0	476					
		P_2	860					
Ryegrass (Unassoc.)	C_0	P_0						394
		P_2						615
Cocksfoot (Unassoc.)	C_0	P_0						337
		P_2						472
Browntop (Unassoc.)	C_0	P_0						566
		P_2						891

yield with increasing potassium application from K_1 to K_2 when associated with cocksfoot (Figure 2).

Percent potassium of grass and clover is shown in Table 3. In the absence of applied nitrogen the grasses tend to absorb potassium in excess of their apparent minimum demands. When grown in association the potassium contents of both ryegrass and browntop increase as the K level is increased in the absence of applied N, but such an increase in percent K is not nearly so evident in cocksfoot. Clover growth appears to have been limited by potassium and, therefore,

shows much less evidence of luxury consumption except in the N_2K_2 treatment of the ryegrass association and in low N, high K treatments of cocksfoot associations.

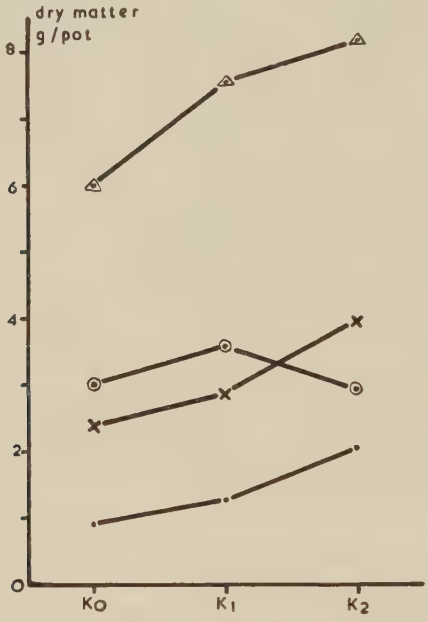

Fig. 2. Effect of K treatment on dry-matter yield of clover grown alone and in association. Average of three N levels.

△ Grown alone ⊙ With cocksfoot
× With browntop • With ryegrass

Table 4 shows the potassium uptake by grasses and clover when grown together and alone. When grown alone, browntop (av. of all treatments 82.2 mg) absorbed more potassium than did correspondingly treated pots of the other species – ryegrass 69.5 mg, clover 68.2 mg and cocksfoot 65.4 mg. This order was not reflected in competition by the grasses, the average K uptake by clover being 23.8 mg in browntop associations, 11.6 mg in ryegrass associations and 31.7 mg when grown with cocksfoot.

It may be seen that the response in K uptake by clover to potassium application was similar throughout the three associations, the G × K interaction on dry-matter yield not being reflected in K

TABLE 3

Percent potassium (K) of oven-dried grass and clover							
Associated grass	K treatment	Clover			Grass		
		N_0	N_1	N_2	N_0	N_1	N_2
Ryegrass	K_0	0.79	0.76	0.73	0.91	0.72	0.63
	K_1	0.79	0.91	0.84	1.19	0.88	0.70
	K_2	0.74	0.81	1.10	1.32	1.13	0.79
Cocksfoot	K_0	0.85	0.87	0.80	0.93	0.84	0.71
	K_1	1.06	0.99	0.75	0.92	0.84	0.74
	K_2	1.45	1.25	0.96	0.99	0.79	0.92
Browntop	K_0	0.78	0.68	0.70	0.87	1.08	0.83
	K_1	0.94	0.72	0.70	1.26	0.85	0.80
	K_2	0.73	0.88	0.80	1.40	0.95	0.85
Clover (Unassoc.)	K_0	0.82					
	K_1	0.89					
	K_2	1.08					
Ryegrass (Unassoc.)	K_0				0.78	0.72	0.64
	K_1				1.18	1.01	0.83
	K_2				1.34	0.96	0.86
Cocksfoot (Unassoc.)	K_0				0.71	0.75	0.64
	K_1				1.06	0.74	0.87
	K_2				1.13	0.88	0.74
Browntop (Unassoc.)	K_0				0.81	0.79	0.70
	K_1				1.03	1.03	1.16
	K_2				1.48	1.15	1.15

uptake by clover because of the high K content of clover in cocksfoot associations receiving applied potassium.

DISCUSSION

In the cut pots of the phosphorus trial and in the potassium trial, the large response of clover to phosphate and potassium respectively, shows the importance of competition for these nutrients in the associations. However, in the uncut pots of the phosphate experiment, it has been seen that phosphate application increased clover production greatly in browntop associations, to a lesser extent in cocksfoot associations and gave no increase in ryegrass associations. It is clear, therefore, that some mechanism other than nutrient

TABLE 4

Potassium uptake (mg K × 10) of grass and clover per pot							
Associated grass	K treatment	Clover			Grass		
		N_0	N_1	N_2	N_0	N_1	N_2
Ryegrass	K_0	81	101	31	379	387	405
	K_1	167	73	73	497	548	463
	K_2	197	181	142	523	597	556
Cocksfoot	K_0	258	275	223	292	298	342
	K_1	415	372	232	313	317	391
	K_2	447	388	253	331	423	474
Browntop	K_0	206	167	143	311	460	364
	K_1	279	212	182	431	434	525
	K_2	334	293	328	468	481	527
Clover (Unassoc.)	K_0	491					
	K_1	673					
	K_2	881					
Ryegrass (Unassoc.)	K_0				450	515	512
	K_1				788	778	707
	K_2				732	872	878
Cocksfoot (Unassoc.)	K_0				400	530	579
	K_1				703	588	786
	K_2				754	800	746
Browntop (Unassoc.)	K_0				501	595	625
	K_1				750	813	950
	K_2				1043	1061	1062

competition, presumably light competition, is operative in the reduction of clover yield in these associations. If the reduction in response of clover to phosphate application when uncut was due to simple light competition, clover could be expected to have a higher percent P content the higher the P treatment in the uncut associations. As this was rarely the case, it is evident that a type of light competition by nutrient competition interaction is causing the reduced clover yields in the uncut ryegrass–clover associations. This interaction is believed to be due to light competition, from the fast growing grass, being implemented before phosphate deficiency is felt by the clover. This would limit the energy supply of clover, and much of the available energy would be expended in upward growth with little root exploitation of the soil. During this stage

there would be little increase in phosphate uptake by clover with increase in applied phosphate and at a subsequent stage when phosphate deficiency limits growth of the association there is little difference between P uptake by clover among the P treatments. The reduction in phosphorus uptake by clover with increasing phosphate application on ryegrass–clover associations receiving nitrogen can be explained by this two-stage mechanism, the grass receiving liberal amounts of nitrogen and phosphorus producing a greater light competition in the first stage so as to more than offset the benefit of the extra phosphorus to the clovers in the second stage.

In view of the large response of the cut associations of both trials to increasing application of the nutrients studied, luxury consumption of phosphorus and potassium by the associations in the respective experiments is considered unlikely. The observed tendency towards luxury consumption as the limiting nutrient was applied to the cut pots of each trial is believed to be due to the fact that the first cut of the associations, which was bulked with subsequent cuts for chemical analysis was probably made before nutrient deficiency was critical and luxury consumption could well have been evident at this stage in the pots receiving applied phosphate or potassium. This increase in P or K content with increasing levels of P or K is noted in both trials in clover associated with cocksfoot, in the phosphate trial in clover associated with ryegrass, but scarcely at all in clover grown with browntop. In both trials there is evidence of the apparent luxury consumption of phosphorus and potassium by browntop and to a lesser extent by ryegrass, but little if any such effect is noted in cocksfoot when associated with white clover.

From these observations it may be implied that browntop, at the time of harvest, was limiting clover growth by competition for phosphate and potassium more than was ryegrass, while in the cocksfoot–clover associations, it was probably the grass that was suffering most from nutrient competition.

The lack of a nitrogen by cutting interaction on clover yield indicates that the suppression of clover by nitrogen application was not due to increased light competition from the grasses but was probably the result of increased nutrient competition due to the stimulation of grass growth.

SUMMARY

Competition between three grass species and white clover for phosphorus and potassium were studied in pot trials. Differences in the ability of the individual grasses to compete with clover were noted to be of the same order for both nutrients.

Nitrogen application was shown to increase competition for nutrients by grasses in clover associations without postulating an indirect mechanism acting through competition for light.

Evidence of a light by nutrient competition interaction was observed and its mechanism discussed.

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COMPETITION FOR NUTRIENTS BETWEEN GRASSES AND WHITE CLOVER

II. EFFECT OF ROOT CATION-EXCHANGE CAPACITY AND RATE OF EMERGENCE OF ASSOCIATED SPECIES

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INTRODUCTION

It has been well demonstrated that the Donnan distribution of ions in the soil, plant-root system may control the uptake of cations (Mattson [12]) and anions (Mattson *et al.*[13]) by plants. Elgabaly and Wiklander [5] showed that in accordance with the valence effect of the Donnan distribution, plant roots with a lower cation-exchange capacity could compete more sucessfully with soil colloids for monovalent cations than could higher cation-exchange capacity roots. Drake *et al.*[4] after finding that the cation-exchange capacity of dicotyledons was roughly double that of monocotyledons suggested that the Donnan distribution effect on nutrient uptake may explain why legumes are poor competitors for potassium when grown in association with grass. Later work (Gray *et al.*[7]) tested this assumption and it was found that the potassium uptake of ladino clover was inversely related to the root cation-exchange capacity of the associated grass. Smith and Wallace [18] went further and stated that there should be an inverse equivalent relationship between the C.E.C. of associated plants and the monovalent cation uptake. However, although the Donnan distribution would favour the absorption of monovalent over divalent cations by the lower

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C.E.C. root, there is no valid reason for suggesting an equivalent relationship of C.E.C. with K uptake.

In the measuring of cation-exchange capacity of live roots, Drake *et al.*[4] experienced difficulty in measuring the adsorption of cations on to the root as distinct from the absorption of these ions into the root cells, a process which also liberates hydrogen ions (Robertson and Wilkins[17]) which may be titrated as exchangeable ions. The sorption of cations by plants involves the adsorption from the external solution and the secretion of these ions into the cell vacuole. Robertson and Wilkins[17] link cation absorption with the metabolic accumulation of anions through the liberation of a hydrogen ion with the reduction of cytochrome. There is evidence of such a relationship between respiration and cation uptake. Overstreet and Jenny[15] noted a simple relationship between respiration and cation uptake while Steward and Harrison[19] observed remarkable parallelism between cation and anion absorption in potato discs with respect to both time and oxygen supply of the medium. Further evidence of this metabolic control of cation uptake may be noted in that, under some conditions, the uptake of anions and cations occurs in nearly equivalent amounts (Bear[1], Walker[21]).

It has been shown (Mouat and Walker[14]) that there is considerable difference in the ability of cocksfoot, browntop, and ryegrass to compete with white clover for phosphate and potassium. As the plants in this trial were grown from seed, it is probable that differences in emergence rates would have a considerable effect on the ability of the grasses to compete for nutrients (Blaser *et al.*[2], Pavlychencko[16]).

MATERIALS AND METHODS

Cation-exchange capacity of white clover, short-rotation ryegrass, browntop, and cocksfoot roots was measured by a method based on that of Drake *et al.*[4]. Fresh, washed roots were electrodialysed in a Mattson cell (Mattson[11]) containing cooling tubes as suggested by Hamfield and Alben[8]. In the early stages of dialysis, the temperature of the centre chamber was kept below 40°C by frequent changing of the dialysates. With an e.m.f. of 230 volts, a dialysis of longer than two hours was found necessary.

Dialysed roots were placed in 200 ml *N* KCl, stirred and immediately titrated with 0.02 *N* KOH to pH 7 as measured by calomel × quinhydrone

electrodes. Drake *et al.*[4] considered that the exchange of K for H on the root was instantaneous and titrated the root-KCl mixture 5 minutes after the addition of the roots to the KCl solution. These workers noted that the titre varied considerably with time but stated that this was probably due to the absorption of potassium further into the root. In the present work, it was found that the marginal sorption of K with time was too great to be merely metabolic absorption. It was therefore decided to construct titration curves (K sorbed by root against time) and to endeavour to separate the curves obtained into ad- and absorption. To do this, the continually stirred root-KCl system was kept at pH 7 by additions of KOH from a burette, and readings of time and titre recorded over a period of two hours or more.

On the completion of the titration, the roots were dried for 48 hours at 96°C and weighed. Titration figures were then expressed as milli-equivalents of K sorbed per 100 g of dry roots.

Since it has been shown that cation absorption may be directly linked with the metabolic activity of the cell, some roots were pretreated in order to kill salt respiration. These roots were dialysed and titrated as previously described. They were then placed in a large volume of 0.001 *M* KCN for an hour, a treatment designed to kill salt respiration but not impede the basic respiration (Lundegardh [10]). After redialysis, the roots were titrated, dried, and weighed.

Two series of roots were used in this study; "old roots" from plants grown alone in the potassium competition trial (Mouat and Walker [14]) and "young roots" of plants grown for about two months in sand fed with nutrient solution.

An index of the emergence rates of the three grasses and white clover was obtained by sowing seed of each of the species in 200 marked positions in seedling boxes. The first seedlings to erupt from each position were counted daily and recorded.

RESULTS

A representative time–K sorption curve of the electrodialysed root is shown in Figure 1. The time–K sorbed relationship may be divided into two distinct phases as shown by the curve AB and the straight line BC. The rapid uptake of potassium by the plant roots over the first half hour or more, and the logarithmic relationship of the time and K sorption in the curve AB indicates that the chemical adsorption of potassium by the plant-root colloid must take longer than supposed by Drake *et al.*[4] and Smith and Wallace [19]. Since metabolic absorption of ions by plant roots has been shown to be a linear function of time (Epstein [6], Leggatt and Epstein [9], Steward and Harrison [20]), it is assumed that the line BC

represents active absorption of potassium as distinct from the adsorption curve AB.

It will be noted that the line BC does not follow smoothly from the curve AB, the jump at B indicating that some nutrient-uptake mechanism has been initiated at this point. Similar observations were made with all titrations, although in many, the jump at B was not of such great magnitude and in some the position of B was less well defined. However, in all cases, when the rate of K sorption with respect to time was considered, a constant rate of K sorption was observed following an extraordinary point or scatter of points (B) following a hyperbolic relationship between time and rate of K sorption.

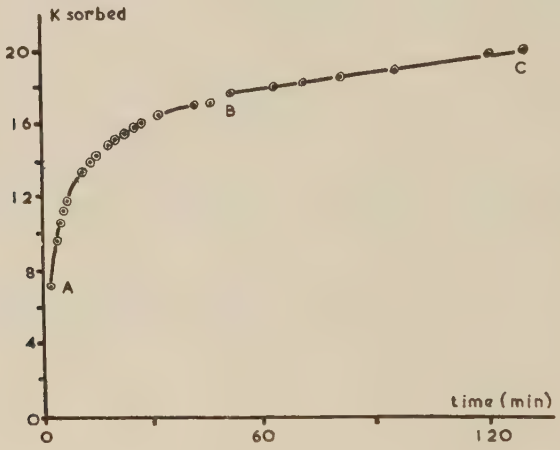

Fig. 1. Time course of K sorption (me/100 g dry weight) by browntop roots.

The increase in rate of uptake at B is probably due to the beginning of metabolic absorption of potassium by the root. Steward and Harrison [20] noted such a time lag before metabolic absorption of bromide ions, and the close parallelism of the absorption of rubidium indicated that the absorption of cations would proceed only after a certain lag period. These workers further noted that the high rate of metabolism to which the subsequent bromide and rubidium assimilation is related occurs some time after the addition of salt solution to the roots.

That the absorption occurred after an appreciable time lag was substantiated when salt respiration of the roots was killed with

potassium cyanide. Time–titration graphs (Fig. 2) showed that the earlier (curvilinear) uptake of potassium was unaffected by the poison but the later absorption, though not entirely checked, was greatly reduced. The inability of respiratory inhibition to completely stop absorption has been previously observed (Butler [3]) and the possible existence of an absorption mechanism not directly dependent on respiration suggested.

Since the evidence indicates that the curve AB in Figure 1 represents adsorption alone and is uncomplicated by accompanying adsorption, the true C.E.C. must be the equilibrium value of AB. Curvilinear regression of the adsorption–time relationship indicated that the adsorption should still be appreciably active after the

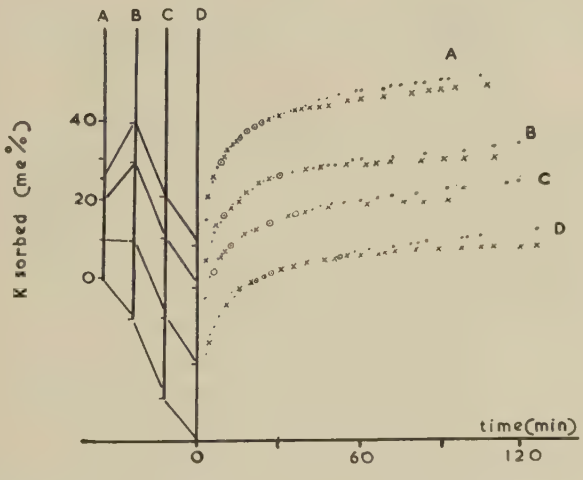

Fig. 2. Time-K sorbed curves of live (•) and KCN-treated (×) roots of white clover (A), ryegrass (B), and cocksfoot (C and D). ⊙ Coincidental points.

initiation of the absorption at point B. However, as absorption subsequent to B in all titrations was linear, it is believed that saturation of the exchange sites does not occur and that the C.E.C. of living plant roots may be represented by the equilibrium of the reactions of adsorption of ions to the exchange sites and absorption from these sites into the plant. The effective exchange capacity is therefore thought to be that value at which the adsorption curve gives way to the absorption line in the K uptake-time course.

TABLE 1

Cation-exchange capacity (me %) of plant roots by different readings. Standard error given in parentheses

Plant roots		Method		
		5-min. reading	Regression of adsorption	Pt. of inflection
White clover	Old roots	16.0 (.96)	39.1 (.92)	35.4 (.63)
	Young roots	22.2 (1.1)	43.0 (1.1)	41.8 (.80)
S.R. ryegrass	Old roots	15.7 (.20)	34.7 (.64)	32.3 (.81)
	Young roots	9.9 (.55)	23.1 (1.0)	20.2 (.10)
Cocksfoot	Old roots	14.4 (.94)	29.8 (1.3)	28.7 (.89)
	Young roots	14.3 (.25)	24.6 (.70)	23.0 (.47)
Browntop	Old roots	10.1 (.50)	17.5 (.53)	15.8 (.50)
	Young roots	7.6 (.18)	15.2 (.07)	14.0 (.15)

Table 1 shows results of C.E.C. determinations as interpreted from the time curves by (a) a five-minute reading, (b) extrapolation of the adsorption curve to two hours, and (c) from the point of inflection of the rate of change of K uptake with respect to time. The difference between old and young clover roots is probably due to a greater proportion of thick tap roots, with comparatively little surface area for their mass, in the older clover plants, while the increase in C.E.C. of grass roots with age may be due to decomposition of dead roots from the root-bound pots, creating more organic-acid exchange sites and a greater adsorbing area per unit weight.

The comparative C.E.C. of these species as determined by the three readings, justifies the earlier, 5-minute reading as a means of obtaining comparative C.E.C. values although it may tend to narrow differences between species.

In order to test the possible errors of the method as a cause of variation of C.E.C. within species, three samples of dialysed short-rotation ryegrass roots were titrated, redialysed and again titrated. Identical curves were obtained in the two titrations and the C.E.C. values did not vary by more than 1 per cent except by the 5-minute reading method, the lower reproducibility of which was no doubt due to difficulty in taking this reading.

The rate of emergence of the plants studied as expressed by the ratio of the sum of the number emerged on individual days of

observation to the product of the number of days of observation and the number finally emerged, was found to be

0.753 for white clover.
0.744 for short-rotation ryegrass.
0.543 for cocksfoot,
and 0.303 for browntop.

DISCUSSION

Results of pot experiments (Mouat and Walker [14]) showed that there was a similarity in competition for phosphate and potassium between grasses and white clover. If, as Gray *et al.*[7] have shown, competition for potassium is a function of root cation-exchange capacity, these results indicate that this property may also influence competition among plants for phosphate. In support of this observation, it has been shown (Mattson *et al.*[13]) that uptake of phosphate by plant roots may be quantitatively related to the Donnan distribution of ions in the root–external solution system. This being so, a system containing two plant-root colloids should show a greater activity of phosphate in the lower-C.E.C. root than in that with the higher C.E.C. Phosphate competition should therefore show a relationship similar to that observed with potassium competition. While a similarity in competition for phosphate and potassium was observed in the present experiments, the relative uptake by associated species was not found to correlate, as expected, with the root C.E.C. of the plants. Figure 3 shows the relative uptake of P and K by the associated grass and clover in the two trials compared with the relative C.E.C. of the associated species. The only apparent cause of divergence of these results from those of Gray *et al.*[7] (also shown in the figure) which give good agreement with that expected from the effect of the Donnan distribution on ion uptake by associated plants, is the wide difference in emergence rates of the species under test. While the American workers minimised differences in early-growth rate by establishing the plants from cuttings, there were large differences in establishment times of some of the species in the present experiment.

Pavlychenko [16] suggested that the degree of competition afforded by a plant depends on its ability to establish more quickly

and exploit the available soil volume before the associated plants. This being so, the ratio of uptake of a limiting nutrient by associated species would be expected to increase with increase in the ratio of the rates of emergence of these species. Figure 4 shows that such a relationship is not applicable to these experiments, discrepancies being due to a relatively high clover : grass uptake ratio by the

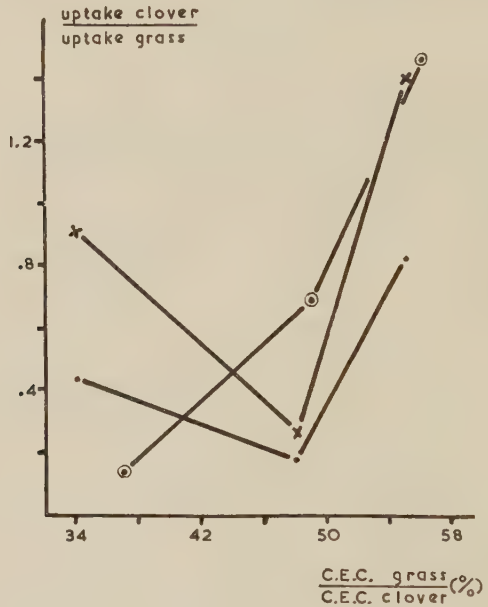

Fig. 3. Clover: grass P (×) and K (•) uptake ratio compared with the grass: clover C.E.C. ratio.
⊙ K uptake and C.E.C. data after Gray *et al.* [7]

cocksfoot associations and a relatively low uptake ratio in the clover–browntop associations. As these divergences are rather the converse of those of the relationship between the uptake ratio and the reciprocal ratio of the C.E.C., it is possible that the competition offered by the associated grass species may be due to both the difference in C.E.C. and the difference in emergence rate of the competing plants; both factors acting independently. The near-constant proportional relationship shown in Figure 5 illustrates the possibility of such effect of the C.E.C. and emergence rate on the outcome of nutrient competition.

Such an empirical relationship is necessarily true only for this particular experiment and is shown purely to illustrate the possible effects of both these factors in determining the outcome of competition for the anion and monovalent cation studied. One condition

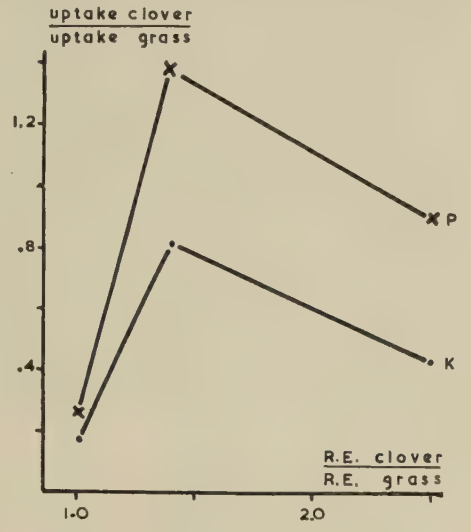

Fig. 4. Clover : grass P (×) and K (•) uptake ratio compared with the ratio of their rates of emergence (R.E.).

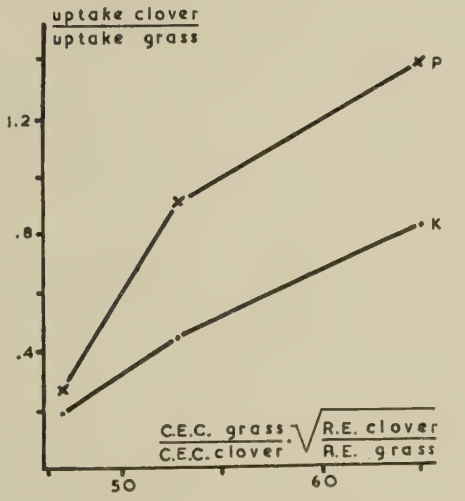

Fig. 5. Clover : grass P (×) and K (•) uptake ratio as a function of the C.E.C. and rate of emergence (R.E.) ratios.

which must greatly modify the relative effect of C.E.C. and emergence rate on the grass–clover balance is the form of the limiting nutrient in the soil, when the nutrient exists primarily in a readily soluble form the effect of establishment rate will be magnified, but when it exists primarily in the exchangeable form or in relatively insoluble phases, the C.E.C. effect might be of greater importance. Another modifying factor is the time of association of grass and clover. It is obvious that given time to acquire ecological equilibrium the association will not be directly affected by the previous establishment rate. It will be mainly in the early development of a grass–legume association therefore, that the effect of establishment rate will be noticed in nutrient competition. This is well shown in ryegrass–white clover and browntop–white clover pastures. A short-rotation ryegrass pasture, even when grazed in the early stages to reduce light competition, will become very grass dominant in the first season, provided that the supply of mineral nitrogen is sufficiently high to meet the demands of the grass, but after the effect of differential establishment rates has been nullified with time, a well-balanced sward is readily maintained with grazing. However when clover is introduced into browntop pastures, despite good establishment, it is rare that a well-balanced grass–clover pasture of any permanence results without heavy fertilization. It is suggested that a probable reason for the lack of success of clover in the browntop pastures, compared with that in the ryegrass pastures, is the greater competition for phosphate, potassium, or perhaps sulphate offered by the browntop as a result of its much lower root cation-exchange capacity, a fact which was not directly observed in the pot trial owing to the great disadvantage of establishment rate experienced by the browntop.

Problems concerned with cocksfoot–white clover balance in pastures do not appear to be due to competition for nutrients as both the C.E.C. and the pot trial results show that cocksfoot is the most compatible of the three grasses studied. The main difficulty in maintaining a well-balanced, productive, cocksfoot–white clover pasture lies in the growth habit and response to grazing of the constituents. Cocksfoot, by assuming a tall, bunchy growth form, quickly suppresses associated clover through competition for light, if the sward is not frequently grazed, but such close grazing is often injurious to the high crowned grass and therefore rather exacting

management must be employed to get maximum production from such an association.

SUMMARY

Cation-exchange capacity of living plant roots was measured by a method involving the measurement and separation of adsorption and absorption of potassium by electrodialysed roots.

The observed similarity in relative competition for phosphate and potassium by grasses with white clover is considered to be due to similarity in the distribution of these ions between roots of differing exchange capacities.

The competition offered by grasses for both phosphate and potassium was shown to be a function of both their root cation-exchange capacity and their rates of emergence.

It is concluded that the severe competition of browntop with white clover may be caused by nutrient competition and is a manifestation of the wide difference in cation-exchange capacity between these plants, while there is little evidence that competition for nutrients is the cause of suppression of clover by cocksfoot.

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ROOT EXCRETIONS IN RELATION TO THE RHIZOSPHERE EFFECT

IV. INFLUENCE OF PLANT SPECIES, AGE OF PLANT, LIGHT, TEMPERATURE, AND CALCIUM NUTRITION ON EXUDATION

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INTRODUCTION

There is little information on the effects of various factors on the exudation of organic substances by plant roots except for the observation by Katznelson, Rouatt and Payne [6] that the amount of amino-acid material exuded by roots was increased by drying the soil to temporary wilting point and subsequent re-wetting. Also, Rovira [11] demonstrated differences in the exudates of different plants and differences due to the age of the plant. The finding by Rovira [12] that the number of micro-organisms which developed on the surfaces of tomato roots was less with plants grown in artificial light than in daylight suggested that light could also influence exudation.

The present investigation arose from the observation that the amino-acid composition of tomato root exudate varied in different experiments. The only adequate explanation was the incomplete control of temperature and light during these experiments which were carried out in a heated glasshouse. This paper describes experiments in which attempts were made to vary temperature, light, and other conditions to study their effects on exudation of sugars and amino acids from roots.

MATERIALS AND METHODS

(a) *Plants*

The plants used were: Tomato, (*Lycopersicon esculentum*) var. South

Australian Early Dwarf; subterranean clover (*Trifolium subterraneum* L.) strain Bacchus Marsh; and *Phalaris tuberosa*.

(b) *Seed sterilization and plant culture*

Surface sterilization of seeds was achieved by shaking for two hours in 7% calcium hypochlorite filtrate. The treated seeds were washed with sterile water and germinated on glucose(1%)–peptone(0.01%)–yeast-extract (0.01%) agar. Germinated seeds which were free of contaminants were transferred to the plant culture tubes containing plant nutrient solution (Hoagland and Arnon [5]). These tubes were 20.5 × 3.2 cm with stainless steel gauze (2mm mesh) suspended 4 cm from the bottom of the tube. During growth the roots were kept in darkness. All the experiments described were performed without aeration as preliminary experiments showed that aeration had little effect on exudation during the first month of growth and increased the difficulty of maintaining sterility. The plant nutrient solution was changed at 14-day intervals.

(c) *Temperature and light intensity*

(i) Temperature. Limited temperature control was achieved by growing plants in ventilated, heated glass boxes (90 × 60 × 60 cm). In the initial experiments these boxes were placed near a northern window of a temperature-controlled room in which the lateral daylight illumination was supplemented by 12 hours per day of overhead lighting with three 500-watt incandescent lamps 60 cm from the plants. These conditions appeared to be unsatisfactory with respect to lighting and subsequent experiments were carried out in the open. This had the disadvantage that the boxes tended to overheat on sunny days but in spite of this a temperature gradient was maintained between the three boxes (see Table 3).

(ii) Light intensity. The use of perforated metal cages over the racks of culture tubes permitted a study of exudation under normal daylight, 40%, and 10% thereof. Temperature measurements showed little difference with treatment.

(d) *Calcium level*

Plants were grown in Hoagland and Arnon's solution and in nutrient solutions containing one tenth, three-fold and ten-fold the calcium level of Hoagland and Arnon's solution. At the lowest calcium level the $Ca(NO_3)_2$ was replaced with $Mg(NO_3)_2$ while the higher levels of calcium were obtained by adding the appropriate aliquot of sterile 0.5*M* $CaCl_2$ to each tube of sterilized normal plant nutrient solution (0.005*M* $Ca(NO_3)_2$), in this way precipitation of calcium phosphate was avoided.

(e) *Inoculation*

Inoculation of germinated seeds which had previously been surface sterilized was carried out to test the effect of micro-organisms on the amino

acids found in the solution surrounding the roots. The inoculum consisted of the washings of unsterilized seeds – for tomato the washings of tomato seed and for clover the washings of clover seed – and this was added to the appropriate tubes immediately after the introduction of the sterilized seeds.

(f) *Chromatography*

The nutrient solution in which the plants had grown was desalted by passage through columns of the exchange resins Zeo-Karb 215 and De-Acidite E. The sugars and neutral substances passed through both columns while the amino acids were adsorbed by the first column (Zeo-Karb 215) from which they were eluted with 0.5*N* ammonia. These two fractions of the root exudate were concentrated at low temperature with a rotary film evaporator (Craig, *et al.*[2]) to a concentration suitable for chromatography. The amino acids were separated and identified with two-dimensional descending chromatography at 20°C with phenol–water (80 : 20) followed by butanol–acetic acid–water (50 : 10 : 10). The amino acids were detected by spraying with 0.2% ninhydrin in alcohol and heating to 90°C for five minutes. The relative concentrations of amino acids in each sample were assessed approximately by rating the intensity of the spot colour from 0 to 5 except in one experiment where particularly dense spots rated 8 and 10. Separation of the sugars was achieved with one-dimensional chromatography by running butanol–acetic acid–water through the paper for three days. The sugars were detected and identified by the orcinol-HCl method of Forsyth [3] and the aniline hydrogen phthalate method of Partridge [7].

(g) *Photography* *

Permanent records of the amino-acid chromatograms were obtained by using Ilford Panchro-line film with a Wratten "B" (green) filter and light from white fluorescent lamps.

RESULTS

(a) *Species of plant*

Although the necessity to apply different amounts of exudate to the chromatograms to obtain satisfactory spot intensities made it difficult for a direct comparison between plants, Table 1 shows that phalaris and tomato exuded considerably greater amounts of amino acids than did subterranean clover. Also, the balance of amino acids differed between plants, *e.g.* glutamic acid was abundant in tomato and phalaris root exudate but not in clover exudate while glycine was a major constituent of phalaris exudate but not of the others.

* This technique was developed by Mr. K. P. Phillips, Waite Agricultural Research Institute, Adelaide, S.A.

TABLE 1

Amino acids (intensity of spot colour) in root exudates of tomato, subterranean clover and phalaris

Plant species	Aspartic acid	Serine	Glycine	Glutamic acid	Asparagine	Threonine	α-alanine	β-alanine	Glutamine	γ-aminobutyric acid	Methionine or valine	Phenylalanine	Leucine	Tyrosine	Cysteic acid	Unidentified spots
Tomato 200 mg root (w/w)	2	2	2	4	1	Tr	2	Tr	2	2	1	–	1	–	–	–
Sub. clover 500 mg root (w/w)	1	1	1	–	Tr	Tr	2	1	1	Tr	2	–	Tr	–	–	1
Phalaris 250 mg root (w/w)	3	2	4	4	1	1	2	1	2	1	1	–	1	–	1	*

* Four faint spots (Tr. only)

(b) *Light*

Experiments in which plants were grown at three light intensities, viz 100, 40, and 10 per cent of full daylight showed that light intensity had a marked effect on the exudation from clover roots. Table 2 shows that the concentration of serine, glutamic acid and

TABLE 2

Effect of light on the exudation of amino acids (intensity of spot colour) from tomato and subterranean clover

Light intensity	Aspartic acid	Serine	Glycine	Glutamic acid	Asparagine	Threonine	α-alanine	β-alanine	Glutamine	γ-aminobutyric acid	Methionine or Valine	Phenylalanine	Leucine	Tyrosine	Cysteic acid	Unidentified spots
Tomato 1.5 mg root (d.w.)																
100%	1	Tr	Tr	1	–	–	1	–	2	–	1	5	3	–	–	
40%	1	1	1	1	–	–	Tr	–	1.5	–	–	4	2	–	–	
10%	–	2	–	1	4	–	1	1	Tr	–	–	3	1	–	–	
Sub. clover 20 mg root (d.w.)																
100%	2	3	Tr	3	Tr	0.5	3	Tr	Tr	1	1	1	2	–	1	Tr
40%	1	1	Tr	Tr	–	Tr	1	Tr	Tr	0.5	0.5	1	1	–	2	Tr
10%	1	1	Tr	Tr	Tr	Tr	0.5	–	Tr	1	0.5	1	0.5	–	1	Tr

α-alanine was considerably less at the lower light intensities. With tomato there was a decrease in the amounts of aspartic acid, glutamic acid, phenylalanine and leucine but an increase in serine and asparagine with decreasing light.

(c) *Temperature*

The first experiments were conducted with tomato in the cool house in two glass boxes – one at room temperature (mean min. and max. 15° and 21°C respectively) and the other heated (mean min. and max. 21° and 31°C respectively). The results (Table 3a) showed that exudation increased at the higher temperature although generally growth was more etiolated and the dry weights of roots less.

These results were substantiated in later experiments in full sunlight covering a wider temperature range (Plate 1 and Table 3b). These experiments with three temperature-controlled glass boxes were carried out in June and July. Although the mean number of hours of bright sunshine per day during these months was only 3.6, light intensity was quite high on many days of light cloud cover. Each plant species was tested simultaneously at the three temperatures to avoid differences in light incidence.

Photographs of one-dimensional chromatograms (phenol–water solvent) of exudate from tomato and subterranean clover at two ages grown at different temperatures (Plate 1) show that the amount of exudate per unit weight of root and also per plant increased markedly with temperature.

By using the two-dimensional chromatography it was possible to demonstrate that there was not simply a general increase in exudation with temperature rise but that the balance of amino acids altered (Table 3). Large amounts of asparagine were exuded by clover roots at the higher temperatures while with tomato asparagine was also increased but to a lesser degree. In all cases the amounts of glutamic acid exuded increased considerably at the higher temperatures. Table 3 also shows that exudation was greater with the younger plants.

An examination of the sugar fractions of the exudate showed that no detectable amounts were released from clover at any temperature and with tomato glucose was detected only at the higher temperatures. Some constituent capable of preventing colour

TABLE 3

Effect of temperature on the exudation of amino acids (intensity of spot colour) from roots of tomato and subterranean clover

Plant and age	Mean temperatures (°C)		Mean dry weight/plant		mg root/spot (wet weight)	Aspartic acid	Serine	Glycine	Glutamic acid	Asparagine	Threonine	α-alanine	β-alanine	Glutamine	γ-aminobutyric acid	Methionine or Valine	Phenylalanine	Leucine	Tyrosine	Cysteic acid	Unidentified spots
	Min.	Max.	Tops mg	Roots mg																	
(a) *Experiment 1*																					
Tomato, 0–2 wk.	15	21	3.97	0.66	200	2	2	1	2	Tr.	Tr.	1	Tr.	1	2	Tr.	1	1	–	1	–
	21	31	3.01	0.47	200	4	3	2	5	2	1	2	Tr.	5	3	1	Tr.	2	–	1	–
(b) *Experiment 2*																					
Tomato, 0–2 wk.	16	30	4.30	0.79**	100	4	1	1	2	Tr.	Tr.	1	Tr.	Tr.	1	Tr.	–	Tr.	–	–	Tr.
	24	36	3.67	0.39	100	3	3	2	3	2	1	2	Tr.	2	2	1	–	Tr.	–	–	Tr.
	32	41	3.77	0.42	100	4	4	3	5	3	2	4	1	1	3	2	Tr.	2	Tr.	–	Tr.
Tomato, 2–4 wk.	16	30	7.70	1.17*	100	1	Tr.	Tr.	1	–	–	Tr.	–	Tr.	–	Tr.	–	1	–	–	Tr.
	24	36	6.40	0.73	100	1	Tr.	1	1	–	–	Tr.	1	Tr.	Tr.	Tr.	–	Tr.	–	–	Tr.
	32	41	6.30	0.50	100	3	3	1	1	3	Tr.	2	1	1	2	1	Tr.	1	Tr.	–	Tr.
Clover, 0–2 wk.	15	31	6:23**	1.05**	200	1	1	1	2	–	–	1	Tr.	–	Tr.	Tr.	–	Tr.	–	–	Tr.
	21	35	5.74**	0.70**	200	2	1	–	3	2	Tr.	1	–	–	–	1	–	Tr.	–	–	Tr.
	28	40	4.22	0.61	200	4	4	–	5	10	4	4	2	1	3	4	2	4	1	–	†
Clover, 2–4 wk.	15	31	8.22*	1.11*	200	Tr.	1	–	1	–	–	1	Tr.	–	–	1	Tr.	Tr.	–	–	Tr.
	21	35	6.47	0.86	200	Tr.	1	1	1	2	–	1	–	–	–	1	1	Tr.	–	–	Tr.
	28	40	5.43	0.80	200	5	2	2	4	8	Tr.	3	2	1	2	3	2	3	–	Tr.	††

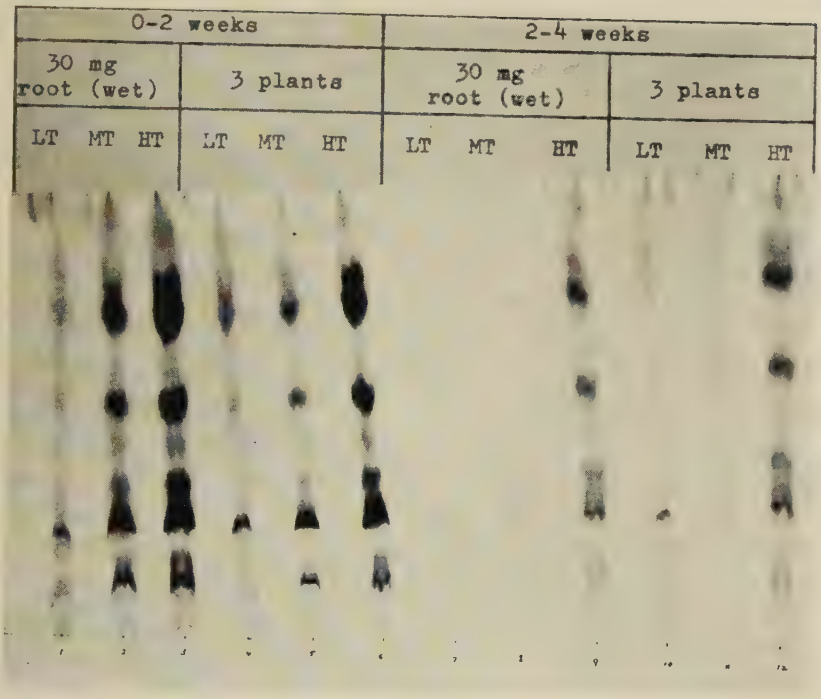
† Five unknown spots with intensities of 2; two spots with intensities of 1.

†† Two unknown spots with intensities of 2 and 3 respectively; one spot with intensity of 1; four spots in trace amounts.

** Significantly different at 1% level from unmarked weights.

* Significantly different at 5% level from unmarked weights.

(a) TOMATO



(b) SUBTERRANEAN CLOVER

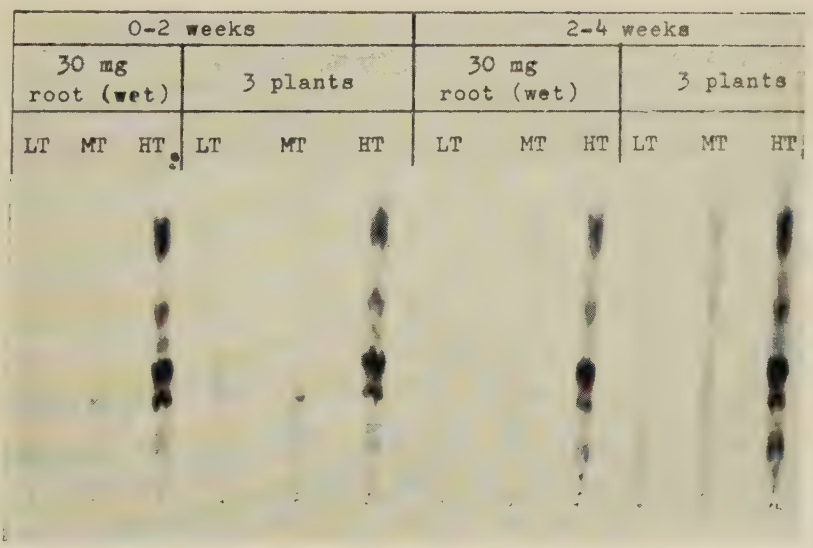


Plate 1. Chromatograms of the amino acids exuded by the roots of tomato and subterranean clover grown at three temperatures.

LT = Low temperature
MT = Moderate temperature
HT = High temperature

} See Table 3 for details

TABLE 4

Effect of calcium on amino-acid exudation (intensity of spot colour) by tomato, subterranean clover and phalaris roots (0–2 weeks)

Plant	Ca level	mg root/spot (wet weight	Aspartic acid	Serine	Glycine	Glutamic acid	Asparagine	Threonine	α-alanine	β-alanine	Glutamine	γ-aminobutyric acid	Methionine or Valine	Phenylalanine	Leucine	Tyrosine	Cysteic acid	Unidentified spots
Tomato	0.0005*M*	200	2	1	1	4	Tr.	Tr.	2	1	2	2	1	–	1	–	Tr.	Tr.
	0.005*M*	200	2	2	2	4	1	Tr.	2	Tr.	2	2	1	–	1	–	Tr.	Tr.
	0.015 *M*	200	3	2	2	4	1	Tr.	2	1	2	1	Tr.	–	1	–	Tr.	Tr.
	0.05 *M*	200	4	3	2	4	2	Tr.	2	Tr.	4	2	1	–	1	–	Tr.	Tr.
Sub, clover	0.0005*M*	500	2	1	1	3	Tr.	–	3	2	1	Tr.	3	–	Tr.	–	Tr.	1*
	0.005 *M*	500	1	1	1	1	Tr.	Tr.	2	1	1	Tr.	2	–	Tr.	–	–	1
	0.015 *M*	500	2	2	1	3	3	Tr.	3	1	1	3	1	–	1	–	1	1
	0.05 *M*	500	2	2	1	2	1	–	2	1	1	1	4	–	1	–	1	2
Phalaris	0.0005 *M*	250	4	5	5	5	1	2	3	–	1	2	1	–	1	–	Tr.	Tr.
	0.005 *M*	250	3	2	4	4	1	1	2	1	2	1	1	–	1	–	1	Tr.
	0.05 *M*	250	4	3	4	3	2	1	2	2	Tr.	2	1	–	1	–	1	Tr.

* Unidentified spot located between methionine and phenylalanine.

TABLE 5

Effect of micro-organisms on the amino acids (intensity of spot colour) present in nutrient solution surrounding roots

Plant	Stage of growth	Sterility	mg root/spot (wet weight)	Aspartic acid	Serine	Glycine	Glutamic acid	Asparagine	Threonine	α-alanine	β-alanine	Glutamine	γ-aminobutyric acid	Methionine or Valine	Phenylalanine	Leucine	Tyrosine	Cysteic acid	Unidentified spots
Tomato	0–2 wk.	Sterile	200	1	1	1	3	–	–	2	–	–	1	–	–	1	–	–	1
		Non Sterile	200	1	2	1	2	Tr.	–	2	–	Tr.	–	Tr.	–	1	–	Tr.	Tr.
	2–4 wk.	Sterile	200	2	1	1	5	2	Tr.	3	–	4	1	2	1	3	–	–	4
		Non Sterile	200	1	Tr.	1	1	1	–	1	–	4	–	1	1	1	–	–	2
Clover	0–2 wk.	Sterile	400	1	1	–	3	1	–	4	1	1	–	1	1	1	–	1	3
		Non Sterile	400	2	Tr.	–	3	–	–	3	2	–	1	1	1	1	–	1	1
	2–4 wk.	Sterile	400	–	1	1	1	–	–	1	1	2	–	1	–	–	–	–	1
		Non Sterile	400	1	1	1	2	–	–	2	1	3	–	1	–	–	–	–	1

development on the paper following spraying with orcinol-HCl was present in all exudates; the area of this spot decreased in the exudate from plants grown at the high temperature. Although these components were not identified they apparently had sufficient buffering capacity to prevent the acid hydrolysis of the cellulose during the heating which follows spraying with orcinol-HCl reagent. This property has been reported for some amino acids, (Porath and Flodin [8]), but in the exudate fraction studied for sugars the spots must have been due to substances capable of passing through cation- and anion-exchange resins.

(d) *Calcium*

The results in Table 4 show that calcium nutrition had no consistent effect on exudation. Maximum exudation of amino acids from tomato occurred at the calcium level equivalent to ten times that in Hoagland and Arnon's solution but with clover and phalaris no such trend was apparent.

The level of calcium affected plant growth only slightly with higher mean weights of tops and roots occurring in the normal Hoagland and Arnon solution with clover and phalaris and at three times this level of calcium with tomato. No sugars were detected in the exudates but a substance possessing buffering properties was present, the size of this spot increased with calcium level.

(e) *Presence of micro-organisms*

The presence of micro-organisms on and around the roots of tomato reduced the free amino acids in the solution during the two- to four-week period of growth but there was little effect on subterranean clover (Table 5).

DISCUSSION

The marked variation in both the amounts and nature of amino acids exuded by the same plant species under different conditions illustrates the importance of complete control of growing conditions if reproducible results are to be obtained. One of the difficulties of glasshouse experiments is that such control is not possible as both temperature and light fluctuate considerably. This can be

overcome in growth chambers but then the light intensity is generally of the order of 1000 foot candles (cf. open sunlight between 8,000 and 10,000 f.c.) which would greatly reduce the amounts of exudates.

These results lead to the question of the mechanism of exudation — is it an uncontrolled leakage from root cells or is it an essential part of the root metabolism in the uptake of nutrients? The mechanism is obviously affected by temperature and light, the former could influence cell metabolism and permeability and nutrient uptake, while the latter will affect mainly metabolism. The resemblance of the spectrum of amino acids in exudate to that of the root sap led Tesar and Kutacek [14] to postulate that exudation is an exosmosis effect although the actual balance of amino acids in the exudate and sap differed. An examination of the free amino acids in roots in the present work often showed a marked discrepancy between the internal and external amino acids which suggests that exosmosis by the whole of the root is not the only mechanism.

There is considerable evidence (Steinberg [13], Coleman [1], and Possingham [9]) that mineral nutrition influences the levels of unbound amino acids in the above-ground portions of plants; generally, mineral deficiencies lead to a relative accumulation of amino acids. From this it might be expected that the amino acids in the roots will be affected, which in turn could influence the composition of the exudates. In fact if temperature and light affect the mineral nutrition of plants this may be the mechanism by which they influence exudation.

In view of the effect of calcium in increasing the numbers of bacteria and fungi in the rhizospheres of subterranean clover and *Paspalum dilatatum* (Rovira [10]), and the importance of calcium nutrition in the legume–rhizobium association (Harris [4]), it was surprising to find that calcium level had so little effect on amino acid and sugar exudation. It could well be, however, that the exudation of growth factors and other organic substances is important in the stimulation of micro-organisms and it is the exudation of these substances which is mainly affected. Also, the lowest level of calcium tested was not sufficient to cause obvious deficiency symptoms although root and top weights were less than at the higher levels.

The importance of root exudates in stimulating microbial development in the rhizosphere is now well established and from the above

results it is obvious that plant species and growing conditions will exert a strong influence on the microflora. This was demonstrated in parallel studies (unpublished) in which it was found that the numbers of bacteria in the rhizosphere of tomato were reduced by 60 per cent when plants were shaded to 40 per cent of full sunlight. Comparisons of the rhizosphere effects of different plants have been made in earlier work but the validity of these comparisons is questionable in the light of the results presented in this paper. Unless the plants are grown under identical conditions of light, temperature, and soil, and harvested at similar stages of development such comparions must be interpreted with caution.

SUMMARY

The exudation of amino acids by the roots of tomato, subterranean clover, and phalaris growing in nutrient solution under different environmental conditions has been studied. The amounts and balance of amino acids exuded by the different plants differed — phalaris and tomato exceeding clover. Exudation was greater from the plants during the first two weeks of growth than in the second two-week period.

Light intensity had a marked effect on the exudation of amino acids by clover, the amounts of serine, glutamic acid and α-alanine in the exudate were less at the lower light intensities. In tomato root exudate aspartic acid, glutamic acid, phenylalanine and leucine decreased with decreasing light but serine and asparagine increased. Higher temperatures increased the amounts of amino acids exuded by tomato and clover roots, the latter released large amounts of asparagine at the higher temperatures. The amounts of glutamic acid from both plants increased considerably with temperature. Calcium level and aeration had little effect on exudation but the presence of micro-organisms influenced the concentration of amino acids in solution surrounding tomato roots.

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"BRUSONE" DISEASE OF RICE IN HUNGARY

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INTRODUCTION

The rice-growing area in Hungary has been greatly increased since World War II, mainly due to the fact that it has been found that the ("szik") alkali soils, which cover a considerable part of the Great Hungarian Plain and which were hitherto for the most part little better than waste land, can be used for paddy growing.

Successful paddy growing has been checked however, and its future prospects have been seriously threatened by the appearence of blast disease ("brusone", "aki-ochi"). In 1949, 1954, and 1955 brusone caused heavy damage, especially on those "szik" alkali soils which now contain no calcium and sodium carbonate *in the surface layer* which has became slightly acid, and on meadow soils, while in other years such as 1950, 1951, 1952, 1956 and 1957, the disease did not occur at all.

The elucidation of the aetiology of the disease is of vital importance to its prevention.

In Hungary, as in other rice-growing countries, various agencies, including micro-organisms, unfavourable climatic conditions and physiological disorders, have been suggested as being the cause of the disease. Those who consider the disease to be due to a microbial pathogen still consider the fungus *Piricularia oryzae* Cav. to be responsible for the disease. Those who favour the hypothesis that the disease represents a physiological disorder suggest that it is due to unfavourable soil conditions. The effect of unfavourable weather conditions in promoting the development of the disease has been generally accepted.

Failure to recognize that the work of Pearsall and Mortimer [13] on the oxidation-reduction potentials of waterlogged soils could

provide a basis for a new approach not only to the application of fertilizers in rice growing (Grist [8]) but also to the problems of the physiological diseases of rice, delayed the solution of the brusone problem for more than ten years.

Study of the conditions under which the disease occurs in Hungary and recent investigations by the author seem to support the contention that brusone is a physiological disease of the rice plant.

MATERIAL AND METHODS

Such a complex problem required examination from many different aspects. For this reason the present investigations have included, in addition to ecological observations, an examination of the chemical and microbiological processes of the soil and floodwater, consideration of meteorological data, and a study of the phenology, physiology and histology of the rice plant.

Observations on the occurrence of the disease were carried out in both the rice-growing regions of Hungary, *i.e.* along the river Danube and along the river Tisza and its tributaries.

Soil and water analyses and microbiological studies were carried out on a calcareous soil containing Na_2CO_3, a meadow soil, and an alkali soil which now contains no calcium or sodium carbonate in the surface layer which is slightly acid.

Soil analyses: The following determinations were made. Hydrogen-ion concentration and E_{h7} by glass and platinum electrodes, respectively. Exchangeable and hydrolytic acidity, alkalinity expressed in Na_2CO_3, and content of $CaCO_3$, K_2O, P_2O_5, available and total N, Fe^{++}, Fe^{+++}, and S^{--}.

Analyses of fresh river water and flood water: The following determinations were made. Hydrogen-ion concentration, total and temporary hardness, alkalinity as Na_2CO_3, total solids, oxygen consumption, and content of Ca^{++}, Mg^{++}, K^+, Mn, SO_4^{--}, NO_3^-, NH_4^+, SiO_2, and P_2O_5.

The methods used in making the determinations have been given in previous papers [17] [18] [19].

Microbiological studies: These have included agar-plate counts of aerobic bacteria and qualitative detection of cellulose-decomposing and sulphate reducing bacteria.

Separation by chromatography and detection by ninhydrin were used in the study of the metabolism of amino acids by the plants.

Meteorological observations: Air temperature, soil temperature in the vicinity of the roots, and solar radiation were measured.

Histological studies: Transverse sections of the nodes were studied in unstained preparations from fresh plant material.

RESULTS

Occurrence of the disease

The alluvial soils along the Danube are rich in lime. Here the disease did not appear at all and *Piricularia oryzae* was not observed even in the disastrous year of 1955.

The disease seems to be restricted to the acidic soils developed on the deposits alongside the river Tisza and its tributaries (Fig. 1). In many areas in this region damage frequently reached 100 per cent.

Fig. 1. Paddy areas in Hungary. 1 = brusone-free,
2 = brusone-susceptible area.

There are therefore soils with which the development of the disease seems to be particularly associated ("susceptible" soils) and other soils which are not associated with its development ("immune" soils). Several soil samples from both kinds of soil have been analysed. The result of the analyses are shown in Table 1.

The "susceptible" soils are heavy soils characterised by high exchangeable and hydrolytic acidity and by absence of calcium carbonate in the surface layer. There are, however, differences in the degree of "susceptibility". Analyses of paddy soils in the irrigated areas of the river Tisza region showed that the disease is more liable

TABLE 1

Analyses of the paddy soils, *before flooding*

Farm	Susceptibility of soil	pH		Alkalinity as Na_2CO_3 %	Hydr. acidity ml	$CaCO_3$ in surface layer %	Total * salts %	Rice area No.
		H_2O	KCl					
Soltszentimre . .	Not susceptible	8.6	—	0.14	—	28,0	0.23	1
Szabadszállás . .	,,	8.4	—	0.09	—	27,2	0.07	1
Kunpeszér . . .	,,	8.5	—	0.13	—	21,0	0.10	1
Szunyog	,,	8.6	—	0.10	—	18,0	0.18	1
Kopáncs	Susceptible	6.4	5.5	0.00	6.0	0.00	0.09	2
Vajhát	,,	6.6	5.3	0.00	8.4	0.00	0.11	2
Tiszasüly . . .	,,	6.5	5.3	0.00	10.2	0.00	0.14	2
Karcag	,,	6.4	5.3	0.00	12.5	0.00	0.10	2

* As determined from electrical conductivity measurements.

to occur on the heavier soils at lower elevations and having higher contents of organic matter and total N (Vámos [18]).

In the same field the disease may occur in one part and not in another, the two areas meeting along a straight line (Frank [6]). Sugar beet had been grown the previous year in that part of the field from which the disease was absent whereas the part where the disease occurred had been used for pasture. In another case the disease appeared in streaks corresponding to the positions of the dikes of the previous year.

The disease has also been observed to occur following levelling of the land. Topsoil rich in organic matter and nitrogen was used for levelling. The boundaries of the field received a thicker layer of soil and it was here that the disease first appeared and caused greater damage to the crop. After a period of time which depended on the thickness of the layer of added soil, the disease became less severe.

From these observations on the occurrence of the disease the following conclusions might be drawn:

1. Though the presence of *Piricularia oryzae* and other fungi on the rice plant usually coincides with the occurrence of the disease, it is not caused by them.

2. Unfavourable weather conditions are also not the primary cause of the disease.

3. The cause of the disease appears to be related to certain soil properties, such as the presence of toxic products resulting from microbial activity.

4. The disease is the manifestation of the combined effects of a number of factors.

Investigations into the cause of the disease directed attention to the role of hydrogen sulphide. This compound is produced during the course of biological processes in waterlogged soils, partly from proteins but mainly from the reduction of sulphates.

Microbiological processes in flooded paddy soils. Sulphate reduction

Following flooding of the soil, micro-organisms, especially aerobic bacteria, multiply rapidly, decompose the abundant organic matter and consume oxygen (Figs. 2 and 3). Under anaerobic conditions the number of bacteria producing hydrogen increases. The presence of the following species has been established: *Clostridium werneri* Bergey et al., *Cl. cellulosolvens* Cowles et Rettger, *Cl. dissolvens* Bergey et al. (Plate 1A), and *Cl. omelianskii* Henneberg. These bacteria decompose cellulose with the production of H_2, CO_2 and organic acids (*e.g.* butyric acid).

The hydrogen formed during the decomposition of cellulose is used by the sulphate-reducing bacteria (*Desulphovibrio desulfuricans*

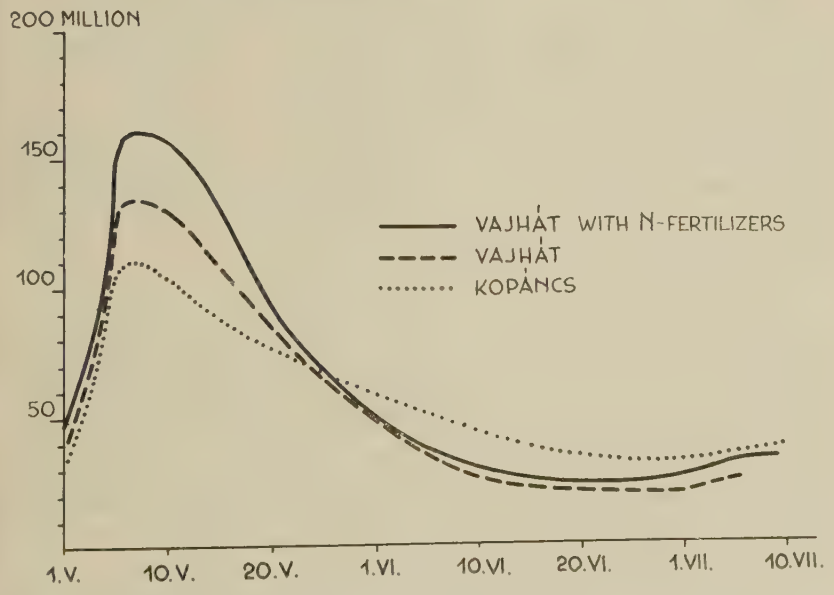

Fig. 2. Changes in the population of aerobic bacteria after flooding (see Table 2).

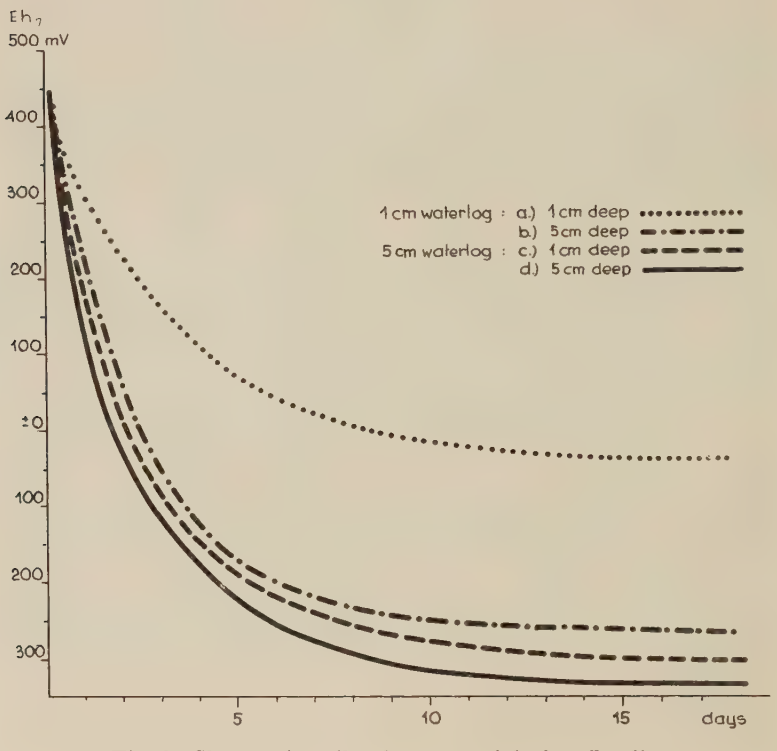

Fig. 3. Changes in soil redox potential after flooding.

Beijerinck; Kluyver et van Niel) as a source of energy (Plate 1B). These bacteria obtain oxygen for utilization of the hydrogen by reduction of the sulphates which are abundantly present in the floodwater and soil (Arany [2], 'Sigmond [15]):

$$4H_2 + SO_4^{--} \rightarrow S^{--} + 4H_2O$$

Takai, Koyama and Kamura [16] also drew similar conclusions.

It has been found that protein-decomposing bacteria also take part in sulphate reduction.

It is considered that these processes, which are reflected in the analytical results given in Table 2, are of vital importance for a better understanding of the physiological processes including nutrient and water uptake and growth of the paddy plant.

Depending on the reaction (pH) of the floodwater, some of the sulphide ions form molecular H_2S, while some form SH^--ions

TABLE 2

Analyses of two "brusone-susceptible" paddy soils, *after flooding*

Soil sample	Days	Eh_7, mV at 5 cm depth	$\frac{Fe^{++}}{(Fe^{++}+Fe^{+++})}$ %	O_2	NO_3-N	NH_4-N	S^{--}
				mg/100 g			
Meadow soil (Vajhát)	0	+ 440	0.0	3.0	0.5	0.5	0.0
	10	— 245	51	—	0.0	3.0	1.4
	20	— 240	78	—	0.0	7.3	4.1
	30	— 245	83	—	0.0	7.6	6.7
	40	— 240	89	—	0.0	8.8	7.0
Alkali soil * (Kopáncs)	0	+ 450	0	2.4	1.0	0.5	0.0
	10	— 235	43	—	0.0	4.0	0.8
	20	— 240	47	—	0.0	4.5	2.2
	30	— 240	53	—	0.0	3.2	3.2
	40	— 245	77	—	0.0	5.7	2.7

* Which now contains no calcium or sodium carbonate in the surface layer which is slightly acid

(Rubentschik [14]). It has been found that the molecular H_2S, which is formed at about pH 7, affects the roots, whilst the SH^--ions, which are formed at about pH 8, are taken up by the roots and damage the tissues of the nodes. In the latter case *Piricularia* seldom appears and the sterile panicles stand rigidly upright ("straighthead"). It has also been observed that in cool, sunless weather the pH value of the floodwater rises, *i.e.* it becomes more alkaline.

The ability of the paddy plant to oxidize H_2S reduces the harmful effects of H_2S:

$$2H_2S + O_2 \rightarrow 2H_2O + 2S$$

The necessary oxygen is, however, only available when the plant is metabolising vigorously. When the weather is cool and sunshine is lacking, carbon assimilation is low and respiration prevails. Under such conditions production of oxygen ceases and the transformation of toxic substances in the soil diminishes. According to Alberda [1] the amount of oxygen passing to the roots decreases considerably when elongation of the stem has ceased, consequently the disease appears only after elongation of the stem is completed. In cool and sunless weather the plant is unable to transform the H_2S in the soil and as a result the roots die. Plants affected by brusone are easily pulled from the soil (Chiappelli [5], Padwick [12]).

Bacteria could not be detected on the roots of diseased paddy plants. Hence the conclusion may be drawn that the toxic substances are lethal not only to the roots and the normal rhizosphere bacteria but also to the saprophytic bacteria which usually colonize necrotic roots. Brizi (Chiappelli [5]) stated that when the symptoms of brusone appear, algae disappear from the water of the ricefield. The observations of the author support this statement.

Following the death of the normal root system adventitious roots develop from nodes higher up the stem. These roots often show negative geotropism (Plate 2A). They are not able to take over the functions of the normal root system since they are in floodwater deficient in nutrients (Vámos [19]) and consequently symptoms of nutrient deficiency and metabolic disturbances ensue (Mitsui, Aso and Kumarawa[9], Okajima and Takagi [10 11], Vámos [19]). Plants showing symptoms of the disease cease growing (Vámos [19]). Disturbances in protein synthesis have been demonstrated by paper chromatography. Fungi make their appearance on the plants only after these metabolic disturbances have supervened (Baba, Iwata and Takahasi [3]) (Plate 2B).

The fungi, *i.e. Piricularia oryzae* and *Helminthosporium oryzae,* are therefore not directly responsible for the considerable damage to the rice crop. Preoccupation with the study of the fungi as the cause of the disease has delayed appreciation of the need to study the relation of the biological processes in the soil to the disease.

The relation between weather conditions and the incidence of brusone

There was considerable less solar radiation in those years when brusone occured than in those years when it did not. In the years 1950, 1951, 1952, 1956, 1957 (Fig. 4) when brusone did not occur the solar radiation and warmth were abundant. The average solar radiation for brusone years and for brusone-free years is given in Fig. 5. Adequate solar radiation enables the plant to produce oxygen by photosynthesis which is in excess of that required for respiration; as a result the H_2S is transformed and the injurious effect prevented. Goto and Tai [7] stated that roots of resistant varieties of rice possess a strong oxidizing power. High oxidizing power of the roots and lower sunshine and heat requirements may be a useful basis for the selection of resistant varieties of rice. Attempts to infect

A

B

Plate 1 A: *Clostridium dissolvens* in paddy soil (2400 ×).
B: *Desulfovibrio desulfuricans* in paddy soil (1600 ×).

A

B

Plate 2 A: Negatively geotropic adventitious rice roots.
B: Spots on rice leaf due to *Piricularia oryzae*.

Plate 3 A:
Transverse section through lowest node of a diseased rice plant (40 ×).

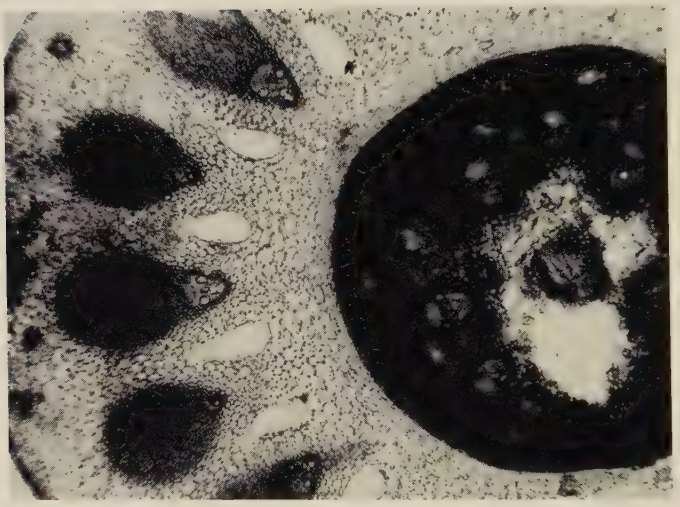

Plate 3 B:
Transverse section through a node of a diseased rice plant (80 ×).

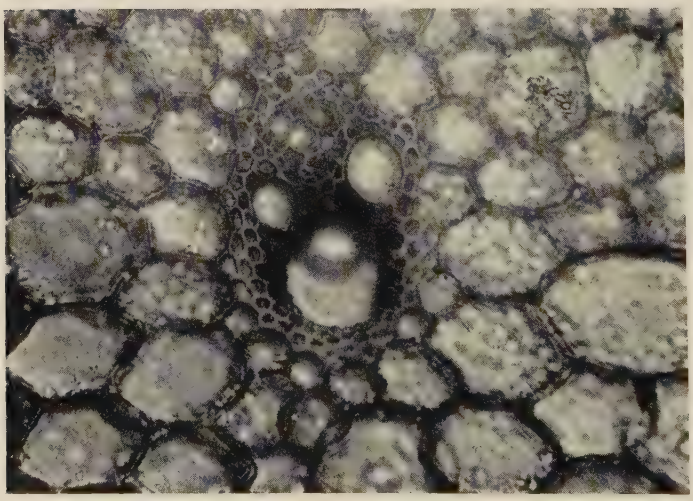

Plate 3 C:
Transverse section through vascular tissue of a diseased rice plant (160×).

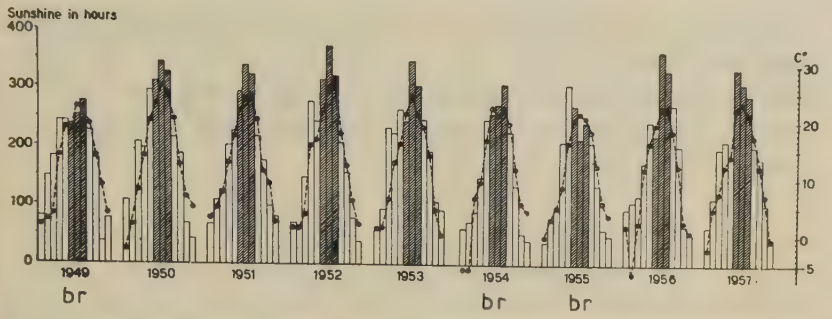

Fig. 4. Solar radiation (histograms) and mean monthly temperatures (curves) at Szeged (University). 1949–1957. br = brusone years.

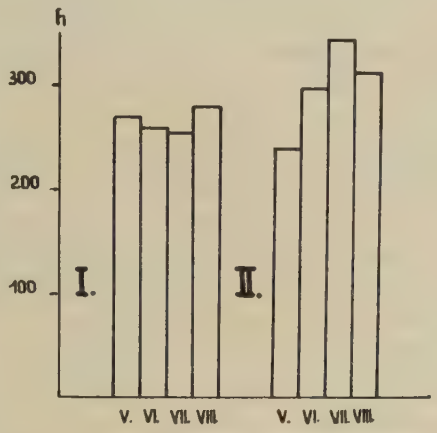

Fig. 5. Average number of hours of solar radiation during the period May to August inclusive, in I: brusone years, and II: brusone-free years.

healthy rice plants with *Piricularia* and *Helminthosporium* using the method of Baldacci and Corbetta [4] were unsuccessful.

Histological studies

The above-mentioned findings have also been confirmed by histological examination.

The most obvious symptom of the disease is the brown discolouration of the tissues of the nodes. The cause of the discolouration is also the cause of the disease.

Histological examination proved that the substance causing the brown discolouration of the tissues got into the plant from the soil

and was distributed in the plant via the water-conducting tissues (Plate 3A, B and C).

The appearance of *Piricularia oryzae* was in all cases preceded by the brown discolouration. The disease known as "straighthead" does not differ histologically from brusone.

DISCUSSION

A schematic representation of the brusone syndrome is given in Fig. 6.

The blast, brusone, straighthead, aki-ochi *etc.* diseases of rice occur on the same soil types in Hungary. Although these diseases are usually considered separately in the literature it is the authors opinion that all these diseases are initiated by a common factor – H_2S. The ultimate manifestation of the disease *i.e.* the ultimate pathological appearance of the plant is governed by the nature of the secondary factors which follow the primary cause of the disease. Consequently H_2S may ultimately produce a deficiency disease following death of roots or it may even cause, according to the present investigations, complete sterility of the flowering rice when it moves from the soil into the air and is than brought in contact with the rice plants in the fogs which form over the rice fields. This is what the Italian and Hungarian peasants call: "the poisonous dawn fog". In some cases the fungi are absent from the injured rice plants whilst in other cases – although the fungi are present – the crop is not damaged; it is the author's opinion that fungal infection is a secondary phenomenon in every case.

The disease has been separated into various forms the symptoms of which are given below.

1. Blast. The plants have a dark-green colour due to abundant N. A third or a quarter of the apical region of the leaves is dry; the characteristic spots of *Piricularia oryzae* infection are present on that part of the leaf which is still fresh but are absent from the leaf sheath. Frequent brown spots – other than those due to fungal infection – indicate potassium deficiency. The plants have healthy roots. The roots growing from the nodes are positively geotropic.

The presence of the fungus does not necessarily damage the crop as plants were noted which – in spite of 50 to 60 spots of *Piricularia oryzae* – were not sterile.

2. Brusone. The roots of plants showing luxuriant development have been completely killed (Chiappelli [5]), the leaves have larger but not more numerous fungal spots (*Piricularia oryzae*). Brown discolouration could always be observed in transverse sections of

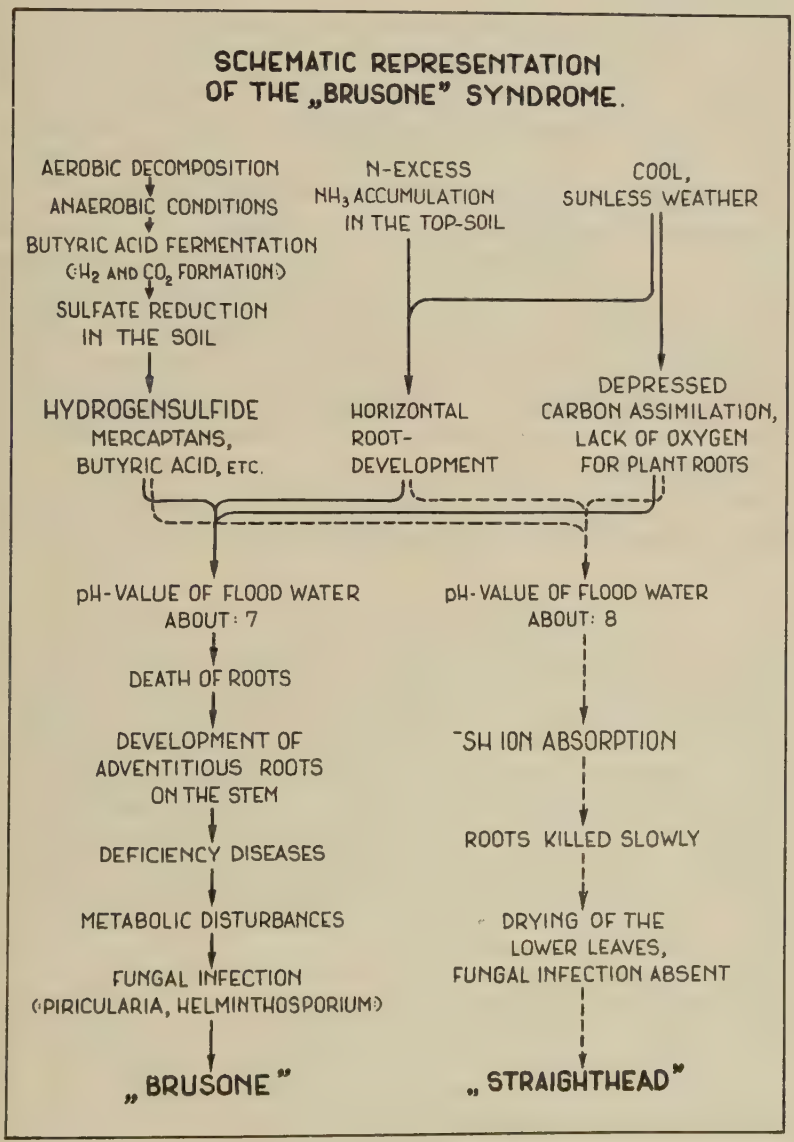

Fig. 6. Schematic representation of the "brusone" syndrome.

the lower part of the stem and nodes. Negatively geotropic roots frequently develop from nodes which are above the surface of the water (auxin deficiency?). The panicle is always empty.

3. Straighthead. For description of the symptoms see Grist [8] and Padwick [12].

The manifestation of the disease may vary due to differing resistance of the plants to the disease, or to dissimilar nutrient conditions and to different meteorological conditions. These different manifestations are probably the basis of the widely differing names ("bianchetta", "gentilomo", "crodature", "carolo", dry leaf disease, Pan-Sukh, "senthal", "akagare", "aki-ochi" *etc.*) which are used for the disease in different countries.

Experiments to prevent the disease with different substances were also carried out. Favourable effect was obtained with $CaCO_3$, CaO, and "red slime" (a by-product of aluminium industry, containing much Ca) but they did not give a complete control. Successful results were obtained, however, with nitrates, especially with calcium nitrate. Nitrates inhibit sulphate reduction and therefore the formation of H_2S.

Results of these experiments and of other experiments on the artificial production of the disease will be described elsewhere.

SUMMARY

Brusone causes considerable damage in Hungary in years with cool, sunless summers. The disease only occurs on certain soil types. The observation, which has been made several times, that healthy and diseased plants may be separated in paddy fields along a straight line, together with histological and biochemical examinations, indicate that the brusone is a soil-borne physiological disease. It has been established by ecological observations and chemical, microbiological, meteorological, histological and physiological investigations that the primary cause of the disease is H_2S. This compound kills the roots of the rice plant. The occurrence of fungi (*Piricularia, etc.*) is merely a secondary phenomenon and is probably connected with disturbance of the nitrogen metabolism of the plant.

The formation of hydrogen sulphide and its toxic effect on the rice plant are promoted by several factors but especially by cool and sunless weather. In such weather the carbon assimilation of the paddy plant is reduced and the toxic H_2S in the soil is not transformed. In such circumstances the disease may cause disastrous damages to rice plants growing on those soils where the disease is particularly inclined to develop.

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PETIOLE ANALYSIS AS A GUIDE TO THE MANURING OF SUGAR BEET

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INTRODUCTION

Although soil analysis is used to assess the nutrient supply available to a crop, Boyd, Garner, and Haines [2] found from sugar-beet experiments that 'whilst there was a general relationship between soil analysis for phosphorus and potash (citric-acid method) and crop response, adjustments to the optimal dressing according to the soil analysis were not of sufficient reliability to be of much practical use.' The problem has also been approached by analysing the living plant to detect any deficiency of a plant nutrient.

The early work to establish the nutrient status of crops and soils was concerned primarily with determining the *total* concentration of the elements present within the plant. By analysing the plant for *soluble* nutrients, the concentration of raw materials within the plant can be given prior to their elaboration into protoplasm and structural tissues and thereby an estimate of the nutrient status of the plant at the time of sampling can readily be made. Under these conditions emphasis in placed upon the nutrient status of the plant rather than upon the nutrient content of the soil.

In recent years Ulrich [6] has developed tissue testing for the determination of the nitrogen requirements of sugar beet under Californian conditions. The following experiment was undertaken as a preliminary investigation into its applicability in Great Britain.

METHODS

The experimental plots were laid out at Harper Adams Agricultural College on a medium loam soil derived mainly from Bunter sandstone. In the

year previous to the experiment the field had been in main crop potatoes for which it had received 20 tons per acre of F.Y.M. and 8½ cwt per acre of a 12.12.15 fertilizer. Yields of ware potatoes from the variety trials carried out in the field ranged from 14.5 to 10.0 tons per acre.

Table 1 shows the soil characteristics at the experimental site.

TABLE 1

Soil characteristics at the experimental site	
Loss on igmition	4.2%
pH	6.65
Available P_2O_5	0.048%
Available K_2O	0.011%

The plots, which were 4 yards by 8 yards in size, were laid out in the form of a 5 × 5 latin square; since the row spacing was 18″, each plot consisted of 8 rows of beet 8 yards long.

Each plot received superphosphate (18%) at the rate of 5½cwt per acre and potash (50%) at 2¾ cwts per acre. The five nitrogen treatments were: –

(a) No nitrogen.
(b) 35 lbs N per acre – equivalent to 2 cwt 'nitrochalk' per acre
(c) 70 lbs N per acre – ,, ,, 4 ,, ,, ,, ,,
(d) 105 lbs N per acre – ,, ,, 6 ,, ,, ,, ,,
(e) 140 lbs N per acre – ,, ,, 8 ,, ,, ,, ,,

Collection of petioles for nitrate-nitrogen determinations

There are two main problems concerning the sampling of the plant for nitrate-nitrogen determinations: (a) which part of the plant to take for analysis and (b) the size and composition of the sample required to give statistically valid and accurate results.

(a) Choice of tissue for testing. The nitrate-nitrogen content at any one time has been found to vary from leaf to leaf of any one plant, and from one part of the plant to another. Ulrich [6] has found that the petiole of the leaf is more suitable than the blade for it reflects the nitrogen supply better. He has found that since there is quite a marked variation from one petiole to another according to the age, the most recently mature leaf is the most suitable one for the purpose of attempting to assess the nitrogen supply.

(b) Statistical considerations. Brown [3] stresses the need for great precision in sampling, - that is a large number of petioles per sample; whereas Ulrich [8] emphasises the need for more samples from an area in order to obtain an estimate of the variability of the nitrogen status of that area. Ulrich suggests that it is better to have a greater number of samples rather than a large number of petioles in one sample.

Most of the variation is from petiole to petiole in the field and not in laboratory analyses. The collections of additional samples from the field or plot is far more valuable than the running of additional analyses on a given sample. In general for plot sampling 20 to 40 petioles are adequate.

Therefore, one sample of about 30 petioles was collected from each plot at each sampling date. For the purpose of sampling, the two outside rows and a yard at either end were discarded, so the effective plot size was 2 yards (4 rows) by 6 yards or approximately 1/400 acre. Care was taken as far as possible to select leaves which were free from virus yellows, which became quite widespread in the crop. The samples were collected once a fortnight from July 29th to September 23rd inclusive, giving five sampling dates.

The petioles were wiped free of any dirt which may have been adhering to them as this could be an important source of contamination with nitrate nitrogen. About 1 to 1¼ inch of the basal part of the petiole was selected and shredded to aid drying. About 80 grams of fresh material on drying in an oven at 80°C gave about 6 to 7 grams of dried plant tissue. The method used for determining the nitrate nitrogen was the rapid colorimetric method, developed by Ashton [1].

In November the discard plants were removed and the plots harvested. Lifting was done by hand as for a normal farm crop. The tops were weighed on the same day as lifting and topping, keeping wilting to a minimum, the leaf surfaces being free of surface water. The roots were put through a washer and then counted and weighed.

RESULTS

(a) *General remarks*

From the time the crop was first examined on July 15th till the end of the sampling on September 23rd the plots were fairly easily distinguishable, especially the no-nitrogen plots which had plants with very stunted foliage. At harvesting the differences in the different treatments were not so distinct.

No attempt was made to measure the occurrence of virus yellows with different treatments. It appeared to become serious at the beginning of August (the second sampling date) when greater care had to be taken when selecting leaves. The general impression was obtained that less virus yellows occurred on the plots receiving high nitrogen applications, but probably this was due to the colouring effect of the nitrogen, for the results obtained by Hull and Watson[5] indicate that excessive nitrogen is associated with an increased loss of sugar through virus infection.

(b) *Nitrate-nitrogen content in the sugar-beet petiole*

The average petiole nitrate-nitrogen values (mean of 5 plots) for different treatments and at different sampling dates can be seen from Table 2.

TABLE 2

Effect of different rates of nitrogen on the nitrate-nitrogen content of the sugar beet petiole at different sampling dates

Treatment, pounds N peracre	Nitrate nitrogen content of petioles in ppm					Mean	L.S.D. (between sampling dates with any one treatment) 661
	July 29	Aug. 2	Aug. 26	Sept. 9	Sept. 23		
0	2625	1247	860	705	605	1208	
35	5675	3570	1845	1230	780	2620	
70	7125	4884	2588	1455	1105	3431	
105	8915	7037	4612	2644	1420	4926	
140	9730	7912	5450	3436	2375	5781	
Mean	6814	4930	3071	1894	1257		
L.S.D. (between treatments at any one date) 1649							

At the first date, the nitrate-nitrogen values range from 9,730 ppm when 140 pounds N per acre was applied to 2,625 ppm when no nitrogen was applied. At the last sampling date the values ranged from 2,375 to 605 ppm. The differences between treatments were found to be statistically significant and the differences between different sampling times to be highly significant. At the later sampling dates and especially with the lower nitrogen treatments, the differences between successive values were smaller than that required for significance. There was found to be an interaction between the treatments and time. This may be explained on the assumption that higher applications of nitrogen to the soil suffer proportionately higher loss through leaching and removal by the plant.

It can be seen from Fig. 1 that at any one date, the values obtained for different treatments do not depart greatly from the regression line. Compared with the other values at the first sampling date, the value for the no-nitrogen treatment appears to be abnormally low if it is assumed that with equal increments of nitrogen, the nitrate-nitrogen values of the petioles should increase in equal increments. It is not possible to give a reason for this since it could be due to so many factors. For example, errors in sampling or analysis, or the effect of weather. This also applies to the mean values obtained from the plots receiving 70 pounds N per acre, on August 26th and September 9th. The changing slope of the regression line illustrates the interaction between the treatments and time.

Fig. 2 shows that with any one treatment there is a gradual fall-off in the petiole nitrate-nitrogen content during the growing

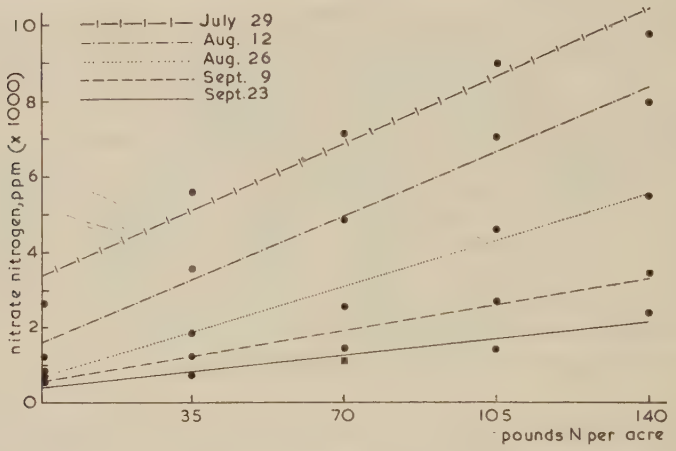

Fig. 1. Regression lines of the variation of petiole nitrate nitrogen with different nitrogen treatments at different dates.

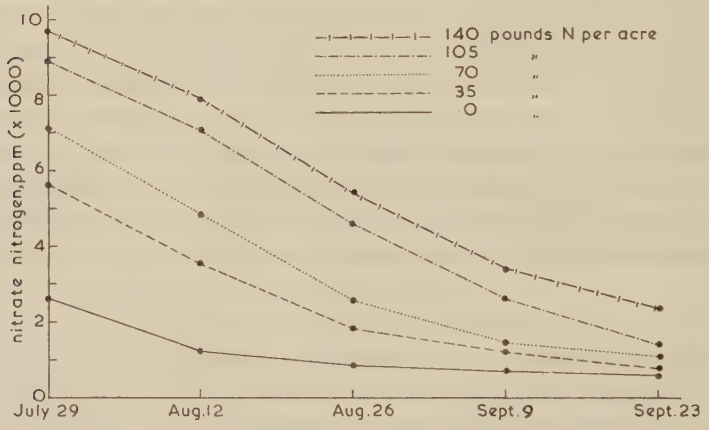

Fig. 2. Variation of petiole nitrate nitrogen at different dates with different nitrogen treatments.

season. As the season progresses, the nitrate-nitrogen values for all treatments tend to become the same.

(c) *Plant numbers*

The number of roots per plot varied from 52 to 69 with an average of 62.3 which would give a plant population of 25,100. It was found that the differences between totals for different treatments were not significant. However, the variances due to the rows and columns

were significant. The variability was probably due to the variation in hoeing.

(d) *Yield of roots*

As shown in Table 3, the yield of roots was found to vary from 15.5 tons per acre with no nitrogen to 18.4 tons per acre with 105 pounds nitrogen per acre. It was found that when the variances of the rows and columns, (since they were comparatively small) were included with that of error, the differences in yield due to treatments were significant at the 1% level. However, if the variances of the rows and columns were kept separate from that of error, then the differences failed to reach the 5% level of significance.

TABLE 3

Effect of different levels of nitrogen on the sugar beet crop

Nitrogen level, pounds N per acre	Root yield, tons/acre	Yield from mean root weight, tons/acre	Tops tons/acre	Roots, number/plot
0	15.5	15.3	10.0	63.0
35	16.8	17.2	11.35	61.2
70	17.4	17.1	13.0	63.8
105	18.4	18.0	14.5	63.8
105	18.4	18.0	14.5	63.8
140	17.7	18.6	15.6	59.8
L.S.D.	1.6	2.1	1.34	N.S.

The high yields can probably be explained partly by the small size of the plot with no wastage of land as headlands as there is with a normal crop; partly due to the high plant population and partly to the high state of fertility of the soil, which is partly explained by the data of Table 1. The plot-yield estimates are not very satisfactory since the number of roots vary from to plot. Possibly a more satisfactory measure of yield is the mean weight of roots, assuming a constant plant population.

(e) *Root mean weight*

An analysis of covariance was carried out on the root numbers and the mean weight of roots, and the regression line was found to be not significant. Therefore the root mean weight for each plot was not corrected for the number of roots per plot.

An analysis of variance of root mean weights showed the differences due to treatments to be only just significant at the 5% level. Although it seems that the different nitrogen treatments had some effect on the yield of roots whether measured by the actual plot yield or the root mean weight, the results should be viewed with caution.

(f) *Yield of tops*

The yield of tops ranged from 10.0 tons per acre with no nitrogen, up to 15.3 tons per acre with 140 pounds nitrogen per acre. Up to 105 pounds N per acre the response was practically linear (an increase of 3/4 tons of tops per acre for each hundredweight of 'nitrochalk'), however the response to the last increment of nitrogen was less. An analysis of variance on top yield showed the effect of different treatments to be highly significant.

DISCUSSION

The object of any technique for assessing the nutrient supply to a crop is to be able to detect a deficiency and to be able to correct it in order to obtain the most economic yield. Therefore, it is generally necessary to correct any deficiency early in the growing season.

Haddock [4] after examining data from various American sources came to the conclusion that, 'plant tissue analyses are useful in appraising the nutritional status of sugar beet plants. The effect of various field treatments on the uptake of nitrogen can be determined with confidence. Frequently the information obtained from plant tissue tests may be useful to commercial growers. But information on the nitrogen-supplying powers of a soil would be much more useful to the sugar-beet grower if it was available before planting.' Whether or not Haddock's conclusions apply in Great Britain it is not possible to say from this single experiment. From the results it is clear that tissue tests do reflect the nitrogen treatments, but it is not possible to say whether or not this technique would be of any use to commercial growers. However, information on the nitrogen-supplying power of the soil before sowing would be of limited use in Great Britain with its very variable weather, for the total uptake and therefore requirements of nutrients in any one season depends to some extent on the weather of that season.

Much of the work on leaf analysis has been done in California where, with their longer growing season the Californian farmers are able to apply nitrogen in the middle of the growing season if necessary and obtain an economic response from it. However in Great Britain there is no advantage in a late dressing of nitrogen for under most soil conditions about $4\frac{1}{2}$ cwt sulphate of ammonia seems to be the optimum dressing whether applied to the seedbed or split for a topdressing.

Assuming from Ulrich's [7] work that the sugar beet plant requires at least 1000 ppm nitrate nitrogen in the petiole to make maximum growth during most of the growing season, and then falling off below 1000 ppm 9 to 11 weeks before harvesting to encourage sugar accumulation; then it can be seen from Table 2 that the no-nitrogen plots fell below the 1000-ppm level 14 weeks before harvesting while the 35 pounds N per acre fell below the 1000-ppm level 10 weeks before harvesting. At the last sampling date, September 23rd, the other three treatments were still above 1000 ppm. If the different dates at which the nitrate-nitrogen values fell below 1000 ppm are responsible for the differences in root yield, which are only just significant anyway, then there would be a case for tissue testing in this country. However much more work would need to be done on the subject.

The fact that the petiole nitrate nitrogen does not fall below 1000 ppm on the plots receiving no nitrogen until mid-August, indicates the high nitrogen supply in the soil after being generously manured for potatoes.

From one year's results, the experiment indicates that the nitrate-nitrogen content of the mature petioles of sugar beet vary not only with the nitrogen treatments but also with time.

The effect of nitrogen on root yields is only just significant whether estimated from plot yields or root mean weight with a constant plant population. Since these results are for a single year on a highly fertile soil they should be viewed with caution.

Since the top yields were so significantly different, it is safe to conclude that the nitrogen treatments had a significant effect on this character.

It should be remembered that these results are from one experiment only and are therefore of limited value. However, in view of the fact that the variation in the petiole nitrate-nitrogen content

with both treatment and time are so significant, further investigation may be of interest.

SUMMARY

During the 1957 growing season an experiment was carried out to see if the Californian method of tissue testing during the growing season to assess the adequacy of the nitrogen supply for optimum growth was practicable in Great Britain. The experiment was laid out in the form of a latin square with the rates of nitrogen application ranging from 0 to 140 pounds per acre in increments of 35 pounds. Petiole samples were collected and analysed at fortnightly intervals from July 29th to September 23rd. The nitrate-nitrogen content of the petioles was found to increase with increasing rates of nitrogen at any one sample date and to decrease during the growing season. On harvesting in November the response in terms of root yield, whether estimated from the actual plot yields, or the root mean weight with a constant plant population, was only just significant. Greater yield responses from the application of nitrogen would probably have been obtained on a less productive soil, for a yield of 15.5 tons of roots per acre was obtained without the use of any nitrogen.

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Plant and Soil XI, no. 1 June 1959

LETTER TO THE EDITORS

An Electrolytic Rocking Percolator

Introduction

The rocking percolation technique described in some of our previous work [2] has been modified. The principle of the modification, which has been applied to respirometers by other workers including Helvey [3], Swaby and Passey [4] Birch and Friend [1] and Wieringa and Kerkhof Mogot [5] consists in the automatically metered electrolytic generation of oxygen within the apparatus to replace exactly the oxygen consumed by metabolic processes. This new electrolytic rocking percolation technique retains all the advantages of the original manometric rocking percolation technique while allowing measurements of oxygen uptake during the breakdown of any compound in soils held at a given partial pressure of oxygen.

Description of apparatus

The main differences between the new percolator assembly and the original manometric rocking percolator assembly (Greenwood and Lees [2], pp. 254–257) are: –

1. The side arm S.A. (Greenwood and Lees [2], Fig. 1, p. 255) has been replaced by a rubber turnover vaccine closure (William Freeman Co. Ltd., Suba-Seal Works, Barnsley, Yorks, U.K.) This enables sampling to be made through the closure with a 5-ml syringe and a 5-inch injection needle without gas exchange between the interior of the percolator and the outside air.

2. The manometer assembly (Greenwood and Lees [2], Fig. 2, p. 256) has been redesigned. The new version, shown in Fig. 1, is essentially a glass U-tube with a tap at the base and having two arms of unequal length, each 1 cm diameter. In one arm (X) there is a constriction D, 0.2 cm diameter, 14 cm from the tap T. A platinum anode A, sealed into the top of X, extends down to D. A copper cathode E sealed in the other arm Y extends from top to bottom of Y. Arm X is connected to a percolator which contains soil and percolate with or without substrate, via tube F (3 mm in diameter) and a similar tube (not shown) fixed to the soil tube of the percolator. The cap of this percolator has no tube fused on to it and acts simply as a seal. This mode of connecting X to the percolator ensures, by virtue of its incorporating a very short connexion, rapid diffusion into the percolator of O_2 generated by electrolysis in X. Arm Y is connected to the cap P.C. of a "compensating

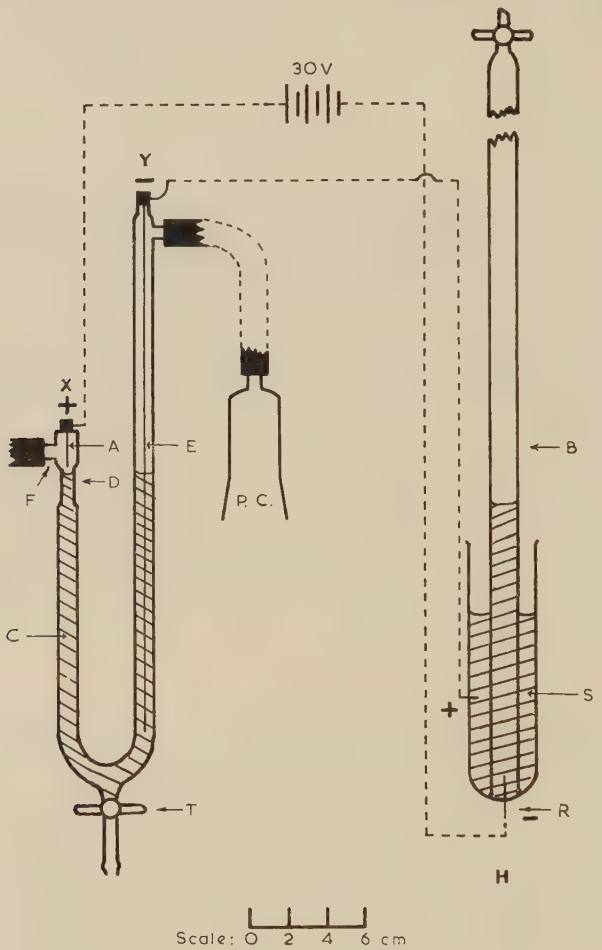

Fig. 1. The electrolytic manometer assembly. X = manometer arm; Y = manometer arm; A = platinum anode; E = copper cathode; D = constriction; P.C. = percolator cap; F = tube; C = saturated solution of copper sulphate in 2*N* H_2SO_4; H = hydrogen cell; R = platinum cathode; S = 2*N* H_2SO_4; B = 25-ml burette.

percolator" containing distilled water of the same volume as the percolate in the percolator containing soil and percolate. Both connexions from manometer to percolators are made with heavy gauge P.V.C. tubing. The electrodes A and E, Fig. 1, sealed in the manometer are connected in series with a hydrogen cell, H, and a 30-volt D.C. supply. The hydrogen cell H contains 40 ml of 2*N* H_2SO_4, S, in a large boiling tube into the base of which a platinum cathode R has been sealed. A 25-ml burette B filled with 2*N* H_2SO_4

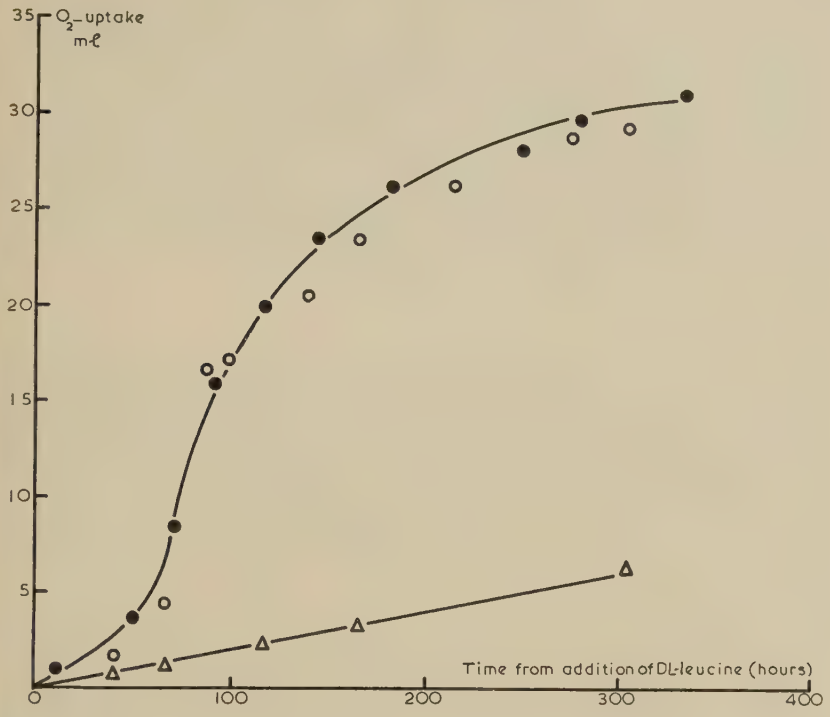

Fig. 2. The O_2-uptake (ml) measured in the electrolytic rocking percolator (–○–) and in the original manometric rocking percolator (–●–), consequent on the percolation of 21 mg DL-leucine dissolved in 50 ml water, through 10 g sieved garden soil. –△–, O_2-uptake consequent on the percolation of 50 ml water through 10 g sieved garden soil.

is inverted over cathode R to collect and measure the H_2 evolved. The anode is a piece of platinum wire sealed into the side of the boiling tube; the O_2 evolved at this electrode is allowed to escape.

As with the manometric rocking percolators, the manometer XY, the percolator caps and the percolators themselves (Greenwood and Lees [2], Fig. 1, p. 255) are fixed to a rocking table. The hydrogen cell for each percolator pair is mounted rigidly some distance away.

Principle of the apparatus

Before the start of any O_2-uptake measurements the manometer XY is filled with a saturated solution of copper sulphate in $2N$ H_2SO_4 almost to the tip of the platinum anode A. When there is an O_2-uptake in the percolator containing soil, *i.e.*, the one attached to arm X, the level of copper sulphate

solution rises until contact is made with the platinum anode. While electrolysis proceeds (current approx. 16 milliamps) O_2 is evolved at the platinum anode A until the level of copper sulphate has been restored to its initial position when contact is broken and electrolysis ceases. As O_2 is evolved at the anode, copper is deposited on the copper cathode E. The volume of O_2 evolved by electrolysis is always equal to the volume of O_2 that has been taken up by soil metabolism so that throughout an experiment the partial pressure of O_2 in the system remains unchanged. The volume of O_2 evolved is found by measuring the H_2 liberated into the burette B; this volume of H_2 liberated being equal to twice the volume of O_2 evolved.

Operation of apparatus

At the start of the experiment, 10 g of sieved dried soil [2] are placed in the soil tube, and 45 ml of water in the main tube, of the percolator connected to arm X. Glass beads (10 g) are placed in the soil tube of the other percolator and 45 ml water added to the main tube. After the vaccine closures have been fitted but before any copper sulphate has been added to the manometer, the percolators are mechanically rocked for a period of 5 days. During this time the initial burst of O_2-uptake due to wetting the soil will have subsided and the endogenous respiration of the soil settled to a constant low value.

A solution of the substrate under investigation (5 ml) is then added to the percolator which contains soil, and 5 ml water are added to the compensating percolator. Rocking is continued for a further 3 hours to establish adequate cation equilibration, after which time a 5-ml sample is withdrawn from each percolator through the vaccine closure. After a suitable delay to allow for temperature equilibration, the manometer is filled to the appropriate level with the solution of copper sulphate from a 250-ml separating funnel attached by a flexible rubber tube to the manometer tap T. The funnel is raised and tap T opened until the copper sulphate solution is just below the platinum electrode A; tap T is then closed again. The reservoir funnel is now disconnected from the manometer, rocking is started, and measurements made of the H_2 evolved in the burette B.

It should be noted that the O_2 determined in this way gives the total O_2-uptake of the soil alone in addition to that due to the decomposition of substrate. Control experiments therefore must be run to determine the endogenous respiration of the soil alone before the O_2-uptake due to the addition of substrate can be determined.

A gas mixture of any composition can be transferred to the percolator by removal of the copper sulphate solution from the manometer and outgassing by evacuation and replacement via tap T; the coppers sulphate solution is subsequently run into the manometer exactly as for the fully aerobic studies described above.

The practical details concerned with securing the caps, *etc.*, described for the original [2] rocking percolators apply also to the new technique. Great care must be taken to ensure that no leak occurs in either the percolators or in the burette.

Sampling of percolator

The percolate may be sampled during the course of experiments in the following way. A 5-inch injection needle connected to a 5-ml syringe is inserted through the vaccine closure of the 'compensating' percolator and a 5-ml sample of the distilled water is withdrawn and discarded, consequently the copper sulphate solution rises up arm Y and falls away from the electrode in X. The desired 5-ml sample is then withdrawn in the same way from the 'soil' percolator, thus causing the level of the manometer fluid to return to its initial position.

Experiments to test the accuracy of the apparatus

It has been found that the apparatus will supply 3.5 ml of O_2 per hour, a far greater rate than is ever demanded in normal experiments on soil metabolism. Moreover control experiments have shown that the diffusion of O_2 from the platinum anode A to the soil/percolate system is rapid enough to ensure that this diffusion does not limit the O_2-uptake of any normal metabolising soil system. Fig. 2 shows that O_2-uptake curves given by the new apparatus agree closely with those given by the manometric apparatus [2].

Summary

The manometric rocking percolation technique has been modified, by incorporating an electrolytic device, so that it can be used to follow the O_2-uptake during the breakdown of any organic compound in the soil at any partial pressure of O_2, whilst still enabling the percolate to be conveniently sampled during the course of the experiment.

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